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Abstract
In most organisms, control of the developmental program involves a regulated transition from maternally supplied mRNAs and proteins to newly synthesized zygotically encoded factors. This phenomena, known as the maternal to zygotic transition (MZT), is observed in a wide range of embryos in the animal and plant kingdoms; in chordates, the MZT typically occurs during midblastula stages, and therefore is often referred to as the midblastula transition (MBT). Early development of most organisms is exclusively maternally controlled, and the zygotic genome of the embryo remains transcriptionally silent until after the MBT, when the transition to zygotic control culminates. Recent work in a number of organisms has identified several genes that are activated prior to the MBT, but whether precocious expression of specific mRNAs is important for later development has not been examined in detail. In this work, I characterize the role of a maternal transcription factor in preMBT transcription, and identify a developmentally significant function for the expression of specific transcripts before the MBT. I identify a class of protein coding transcripts activated before the MBT by the maternal T-box factor VegT, all of which are components of an established transcriptional regulatory network required for mesendoderm induction in Xenopus laevis, including the Nodal related ligands xnr5, xnr6, and derrière and the transcription factors bix4, and sox17α. Phosphorylated Smad2, a hallmark of active Nodal signaling, is present before the MBT and preMBT transcription of xnr5 and xnr6 is required for this activation. Furthermore, preMBT activation of the Nodal pathway is essential for mesendodermal gene expression and patterning of the embryo. Together, this work demonstrates that regulated transcription before the MBT is important for the establishment of dorsal mesoderm and the development of the embryo.

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THE ROLE OF PREMBT TRANSCRIPTION IN XENOPUS DEVELOPMENT

Jennifer N. Skirkanich

A DISSERTATION

in

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DEDICATION

This work is dedicated to my Mom and Dad,

without whom none of this would be

possible.

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ABSTRACT

THE ROLE OF PREMBT TRANSCRIPTION IN XENOPUS DEVELOPMENT

Jennifer N. Skirkanich

Advisor: Peter S. Klein, M.D., Ph.D.

In most organisms, control of the developmental program involves a regulated transition from maternally supplied mRNAs and proteins to newly synthesized zygotically encoded factors. This phenomena, known as the maternal to zygotic transition (MZT), is observed in a wide range of embryos in the animal and plant kingdoms; in chordates, the MZT typically occurs during midblastula stages, and therefore is often referred to as the midblastula transition (MBT). Early development of most organisms is exclusively maternally controlled, and the zygotic genome of the embryo remains transcriptionally silent until after the MBT, when the transition to zygotic control culminates. Recent work in a number of organisms has identified several genes that are activated prior to the MBT, but whether precocious expression of specific mRNAs is important for later development has not been examined in detail. In this work, I characterize the role of a maternal transcription factor in preMBT transcription, and identify a developmentally significant function for the expression of specific transcripts before the MBT. I identify a class of protein coding transcripts activated before the MBT by the maternal T-box factor VegT, all of which are components of an established transcriptional regulatory network

required for mesendoderm induction in *Xenopus laevis*, including the Nodal related ligands *xnr5*, *xnr6*, and *derrière* and the transcription factors *bix4*, and *sox17*α. Phosphorylated Smad2, a hallmark of active Nodal signaling, is present before the MBT and preMBT transcription of *xnr5* and *xnr6* is required for this activation. Furthermore, preMBT activation of the Nodal pathway is essential for mesendodermal gene expression and patterning of the embryo. Together, this work demonstrates that regulated transcription before the MBT is important for the establishment of dorsal mesoderm and the development of the embryo.

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CHAPTER 1: Introduction

Following fertilization, most metazoans undergo a series of rapid and synchronous cleavage divisions characterized by transcriptional silence and the absence of a G phase in the cell cycle (Bachvarova and Davidson, 1966; Graham and Morgan, 1966; Newport and Kirschner, 1982a). This continues until the midblastula-transition (MBT) when the cell cycle slows, cell divisions become asynchronous, cells gain motility, many maternal mRNAs are degraded and zygotic transcription is activated. The factors that coordinate this transition are largely unknown. In recent years, regulated transcription prior to the MBT has been demonstrated in many organisms, suggesting a conserved function for such transcription. In this work, I identify a transcription factor that activates zygotic gene expression prior to the MBT and show that mRNAs transcribed before the MBT are essential for patterning the early *Xenopus* embryo.

A note regarding the MBT

The midblastula transition is a general term often used to describe the period of blastoderm development when the major changes associated with the MZT occur in the early development of an organism. The term itself first appeared in literature in the early 1970's and 1980's as "transition blastuleenne", (Signoret and Lefresne, 1971), and as "midblastula transition", (Gerhart, 1980). Although the events associated with changes in the embryo during the midblastula stages have been independently described in various organisms for nearly a century (Boveri, 1918; Hertwig, 1918; Schonmann, 1938; Dettlaff, 1964; Signoret and Lefresne, 1971; Gerhart, 1980), this term was first used to describe the collection of cellular events including zygotic genome activation (ZGA), slowing of the cell cycle, and acquisition of cell motility, by Newport and Kirschner (1982a). Later, degradation of maternal RNAs was also identified as an event associated with the MZT (Audic et al., 1997). In this thesis, the term MBT refers to the collection of

events occurring during this transition as described in Newport and Kirschner's seminal works (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b), as well as the time during normal development when these events generally occur. In *Xenopus* laevis, "MBT" occurs at the 4000-cell stage, also known as embryonic stage 8.5 (Nieuwkoop and Faber, 1994). In contrast, the term maternal-to-zygotic transition (MZT) refers here to the general phenomenon of transfer of developmental control of the organism from maternal to zygotic, a period which includes the MBT.

Part I: Maternal control of early development

Changes in transcription associated with oocyte maturation and fertilization

During oogenesis, the *Xenopus* oocyte is characterized by robust transcriptional activity that becomes quiescent upon maturation (Wormington and Brown, 1983). Fertilization results in a series of cellular events including the activation of the first cell cycle and cortical rotation. Cortical rotation is a process whereby the cortical or outer-most cytoplasm rotates with respect to the inner cytoplasm, resulting in the translocation of vegetally localized dorsal determinants to the prospective dorsal side of the zygote (Moon and Kimelman, 1998). This crucial event establishes the early dorsal-ventral axis in the *Xenopus* embryo, and will be discussed in detail later in this chapter. Within 90 minutes of fertilization, the first of 12 rapid cleavage divisions occurs.

Cleavage events and the cell cycle after fertilization in the frog.

Most somatic cells undergoing mitotic divisions cycle through four distinct phases known collectively as the cell cycle; this includes a DNA synthesis (S) phase and a mitosis (M) phase, each preceded and followed by a resting gap phase (G1 and G2) (Alberts, 2008). The absence of a G phase in the cleavage stages of *Xenopus* laevis was first reported by Graham and Morgan (1966) in their studies measuring the phases of the cell cycle at various points during development (Graham and Morgan, 1966). The lack of a G phase allows rapid rounds of DNA synthesis and mitosis, taking approximately 23 minutes to complete a cycle. The cell cycle slows after the 12^{th} cleavage division, when the MBT occurs (Graham and Morgan, 1966; Newport and Kirschner, 1982a). These rapid cell divisions are governed by the cyclic activity of mitosis promoting factor (MPF); high MPF activity correlates with M phase, and absence

of activity correlates with S phase (Gerhart et al., 1984; Newport and Kirschner, 1984; Swenson et al., 1986). MPF is a heterodimeric protein composed of Cdc2 and CyclinB, and is regulated by periodic CyclinB degradation as well as inhibition and activation of Cdc2 by Wee-like kinases and Cdc25 phosphatases, respectively (Ferrell et al., 1991; Dunphy, 1994; O'Farrell, 2001). The rapid cycling of preMBT cells appears to be incompatible with activation of cell-cycle checkpoints, and thus the embryo cannot respond to DNA damage until the G phases resumes at the MBT; indeed, DNA damage inflicted prior to the MBT will not stop the rapid cleavages and activate appropriate apoptotic responses until after the MBT (Kimelman et al., 1987; Anderson et al., 1997; Finkielstein et al., 2001).

It is largely thought that chromatin compaction during M phase is incompatible with transcription (Kimelman et al., 1987; Martinez-Balbas et al., 1995); likewise, multiple and rapidly progressing DNA replication forks during S phase make the stable association of transcription factors with DNA unlikely (Wolffe and Brown, 1986). Because of this, one of the proposed explanations for the absence of transcription during cleavage stages has been the inaccessibility of chromatin to transcription factors during both S and M phases. The idea that the rapid cell cycle during cleavage division is responsible for inhibition of transcription is supported by studies from Kimelman and colleagues (Kimelman et al., 1987), who showed that cell cycle arrest during the preMBT period (using cycloheximide) allows precocious RNA synthesis. Similarly, Edgar and colleagues show that injection of cycloheximide into preMBT *Drosophila* embryos results in activation of gene expression before the MBT (Edgar and Schubiger, 1986). Also supporting these ideas is evidence that chromatin assembly and transcription factor complex assembly compete for binding to DNA during the rapid cleavage stages of early development; in fact transcriptional silencing of an exogenous expression plasmid can

be temporarily overcome by preincubation of the plasmid with TBP prior to injection, although this is transient, as the plasmid becomes rapidly chromatinized and silenced (Prioleau et al., 1994).

However, it has become clear more recently that transcription factors can indeed bind to chromatin during rapid cleavage divisions. For example, embryos deficient for the cell cycle regulator Wee-1 still experience rapid cell cycles after the MBT, however the timing of zygotic gene expression is not affected, suggesting that slowing of cell cycle is not required to activate transcription (Murakami et al., 2004). Additionally, regulated transcription does occur at some loci prior to the MBT in *Xenopus* (Yang, Jing et al., 2002; Blythe et al., 2010), although the vast majority of genes remain silent until large-scale genome activation at the MBT. These observations suggest that large-scale transcriptional repression prior to the MBT is regulated by factors other than cell-cycle duration alone.

The nuclear-to-cytoplasmic ratio and control of the MBT

In *Xenopus*, the MBT occurs consistently at the 12th embryonic division. One proposed explanation for the reliable timing of this event has been that it is regulated by a mechanism that counts cell divisions. However, by inhibiting cell division with either cytochalasin B or centrifugation, Newport and Kirschner showed that cleavage per se is not required for the correct timing of the MBT (Newport and Kirschner, 1982a). To examine the theory that the cell regulates timing of the MBT by counting nuclear divisions, they performed partial constriction assays on developing embryos. They used a ligature to constrict a nucleus-free portion of the cytosol from one cell of a 2 or 4-celled embryo; the remaining cells continued to divide, and eventually a nucleus randomly migrated across the ligature boundary to the constricted, nucleus-free cytoplasm. Once

the constricted cytoplasm acquired a nucleus, it began to undergo regular cleavage divisions, and produced a twinned embryo. Intriguingly, the original embryo and the new twin reached the MBT after 12 cleavage divisions each. Even though the nucleus used to create the twinned embryo had experienced several divisions in the original embryo before migrating across the ligature to create the twin embryo, once there, the nucleus went through 12 *additional* cleavage divisions before it reached the MBT. The results of these studies suggest that the events of the MBT are determined not by the number of mechanical cleavages, or even by the number of nuclear divisions, but instead, by the ratio of the volume of nuclei to cytoplasm at each cleavage division.

In these studies, Newport and Kirschner also noted that polyspermic embryos reached the MBT earlier than control embryos, at a rate that corresponded to the amount of excess nuclear material. Additionally, they established that the MBT takes place earlier in embryos that have been injected with exogenous DNA. Based on these results, they proposed that the MBT is triggered by the loss of a cytoplasmic factor, titrated out by the increasing nuclear content at each round of replication. This could explain the coordination of many of the phenomena associated with the MBT; the factor depleted from the cytoplasm at the MBT could be a master (negative) regulator of transcription, cell mobility, and the cell cycle. Indeed, cell-cycle checkpoints, usually not activated in response to DNA damage until the MBT can be induced precociously by altering the nuclear-to-cytoplasmic ratio (Conn et al., 2004). Work in *Drosophila*, zebrafish and starfish has since demonstrated that the nuclear-to-cytoplasmic ratio also controls the timing of the MBT in these organisms (Mita, 1983; Mita and Obata, 1984; Kane and Kimmel, 1993; Pritchard and Schubiger, 1996).

Availability of basal transcription factors before the MBT

The basal transcriptional machinery is present in the *Xenopus* laevis oocyte and early embryos (Veenstra, 2002; Brown, 2004). Additionally, many pathway-specific transcriptional activators are provided maternally (discussed in Part IV of this introduction). Since availability of these factors does not seem to be a rate-limiting step for transcription during cleavage stages of development, it is possible that regulation of these factors does play a role in transcriptional quiescence during the preMBT period.

RNA Polymerase II (RNAP II) is necessary for transcription of protein coding genes in eukaryotes (Hirose and Ohkuma, 2007). RNAP II activity is regulated by phosphorylation of two serine residues within a heptapeptide repeat located in its C-terminal domain (CTD). Phosphorylation at serine 5 (Ser5) of the CTD heptapeptide repeat by TFIIH and Mediators is associated with transcriptional initiation and promoter clearance, but not active elongation; in contrast, phosphorylation at serine 2 (Ser2) by P-TEFb is associated with productive transcriptional elongation (Komarnitsky et al., 2000; Peterlin and Price, 2006; Hirose and Ohkuma, 2007; Egloff and Murphy, 2008). Prior to fertilization, *Xenopus* eggs show similar amounts of phosphorylated and unphosphorylated RNAP II; however hyperphosphorylated forms of RNAP II decrease dramatically within one hour of fertilization (Palancade et al., 2001). Hyperphosphorylated RNAP II then remains undetectable through cleavage stages, until the MBT (Palancade et al., 2001), an observation consistent with findings in other metazoans including *C. elegans* and *Drosophila melanogaster* (Seydoux and Dunn, 1997; Dantonel et al., 2000; Leclerc et al., 2000). Remarkably, by preventing the MBT with the DNA-replication inhibitor aphidicolin, or by advancing the onset of the MBT by creating polyspermic embryos, Palancade and colleagues demonstrated that they could prevent the onset of hyperphosphorylated RNAP II or cause precocious

hyperphosphorylation of RNAP II, respectively (Palancade et al., 2001). This group and others were able to show that although the Ser5 phosphorylated form of RNAP II associated with transcriptional initiation is detectable before the MBT, the Ser2 phosphorylated form is not detected until after the MBT (Palancade et al., 2001; Collart et al., 2009). Collart and colleagues were able to show that Smad-interacting CPSF 30-like factor (Smicl) is required for Ser2 phosphorylation, allowing transcriptional elongation and RNA processing. Interestingly, they demonstrated that Smicl is restricted from entering the nucleus until the MBT, and depletion of Smicl leads to the loss of expression of many genes at the MBT (Collart et al., 2009). Additionally, they found that loss of Smicl leads to a delay in the degradation of several maternal mRNAs, an event usually associated with the MBT, suggesting that these processes may be regulated similarly. Smicl therefore regulates portions of at least two processes associated with the MBT: activation of elongating RNAP II and degradation of maternal mRNAs. However, the regulation of Smicl nuclear localization itself remains unclear. Interestingly, more recent work from our lab has shown that phosphorylated Ser5 and Ser2 forms of RNAP II are present in both pre- and postMBT embryos, a result consistent with transcriptional elongation before the MBT (Blythe et al., 2010).

TATA-binding protein (TBP), a key component of TFIID, is required for basal and active transcription and is considered an essential part of the transcription complex (Hernandez, 1993). TBP mRNA is abundant in *Xenopus* eggs but is only efficiently translated near the onset of the MBT (Bell and Scheer, 1999; Veenstra et al., 1999). These findings suggest that limited access of the early embryo to TBP creates a barrier for preMBT transcription in the embryo. In support of this, pre-loading of an expression plasmid with TBP prior to injection can overcome the transcriptional silencing normally associated with exogenously introduced expression constructs (Prioleau et al., 1994).

However, more recent work has shown that several TBP-like factors are available throughout cleavage stages, suggesting this factor is not limiting in the early embryo (Veenstra et al., 2000; Jallow et al., 2004).

Chromatin regulation during early development

The study of the regulation of chromatin as a mechanism for controlling developmentally relevant gene expression and patterning has received much attention in recent years. When the oocyte becomes transcriptionally silent upon maturation, nucleosomes begin to form more regular arrays, associated with an increase in the affinity of exogenous plasmid to become rapidly chromatinized and a decrease in DNAse I hypersensitivity sites, indicative of condensed, inactive chromatin (Wormington and Brown, 1983; Landsberger and Wolffe, 1997). Rapid DNA replication during cleavage stages requires a large maternal supply of core histones to allow new chromatin assembly at each division; along these lines, enough histones are synthesized during oogenesis to supply the embryo with the material to assemble 13,000 to 16,000 nuclei without zygotic transcription (Adamson and Woodland, 1974; Woodland and Adamson, 1977). These factors establish a repressive chromatin environment in the egg that remains after fertilization and throughout early development. Changing the nuclear-to-cytoplasmic ratio of the cleavage stage embryo by injection of exogenous DNA is thought to induce a premature MBT by titration of a repressive factor (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). Components required to chromatinize DNA, such as histones, present themselves as good candidates for such a factor, through their competition for binding to DNA with transcription factors (Prioleau et al., 1994).

Methylation of DNA at CpG dinucleotides is an important transcriptional regulatory mechanism. Genes bearing this mark are targeted for silencing by the inhibition of association with transcriptional activators, by associating with methyl-CpG-binding proteins (MBPs) which recruit transcriptional co-repressors including chromatin remodeling complexes, or both (Klose and Bird, 2006). Although regulation of genome-wide DNA methylation is an attractive mechanism to explain the switch from preMBT genomic silence to postMBT zygotic gene activation, there has not been a consensus addressing the global methylation state of DNA pre and postMBT. While some reports have noticed a 40% global reduction in methylated cytosine between pre and postMBT stages, indicating a generalized pre/postMBT regulatory mechanism (Stancheva and Meehan, 2000), other reports have not seen a change in genome-wide methylation patterns between pre and postMBT stages (Veenstra and Wolffe, 2001). However it should be noted that neither of these accounts conflicts with temporal regulation of gene expression by DNA methylation at specific promoters. Indeed, Stancheva and colleagues (2002) demonstrated that although demethylation of genomic DNA in embryos approaching the MBT is not uniform, many individual promoters experience a gradual decline in methylation status as they near the MBT (Stancheva et al., 2002).

Additionally, the Meehan group has shown that depletion of a maintenance DNA methyltransferase, Dnmt1, in *Xenopus* embryos leads to the precocious activation of zygotic genes normally activated at the MBT. In this context, Dnmt1 protein presents itself as a candidate for a titratable repressor of gene expression; along these lines, the maternally supplied Dnmt1 mRNA levels decrease as the early embryo approaches the MBT, further suggesting that Dnmt1 may mediate the onset of expression of some genes at the MBT (Stancheva and Meehan, 2000; Stancheva et al., 2002). Strikingly,

gene silencing can be rescued by a catalytically inactive form of Dnmt1, suggesting an additional level of regulation by Dnmt1 (Dunican, Donncha S et al., 2008).

Depletion of the MBP Kaiso also results in precious gene expression before the MBT (Ruzov et al., 2004; Ruzov et al., 2009). Interestingly, although depletion of Kaiso or Dnmt1 advances the expression of some genes, other events associated with the MBT are not changed (Newport and Kirschner, 1982a; Stancheva and Meehan, 2000; Ruzov et al., 2004). This highlights the specific role that DNA methylation might play in regulating the onset of gene expression. Further work is needed in this area to better understand the role that DNA methylation plays in regulating the transition from preMBT silence to postMBT genome-wide activation, particularly to address if this mark is causative of DNA silencing or merely correlative.

Histone modification may also play a role in control of zygotic genome activation. Covalent methyl marks such as histone H3 lysine 4 trimethylation (H3K4me3) and H3K27me3 are associated with activation of transcription and repression of transcription, respectively (Berger, 2007). Recent studies have begun to look at the changes in global histone modifications during development in *Xenopus* and zebrafish (Akkers et al., 2009; Vastenhouw et al., 2010). A genome wide analysis of the development of the closely related frog species *Xenopus tropicalis* failed to detect active or repressive histone modifications prior to the MBT; genes that are activated after the MBT acquire H3K4me3 just prior to or coincident with their expression, and the repressive H3K27me3 mark is not detected until gastrula stages (stage 11) (Akkers et al., 2009). A study in zebrafish had similar findings demonstrating that, on a genome-wide scale, chromatin has neither H2K4me3 nor H3K27me3 marks before the MBT, and then acquires both of these marks at the MBT (Vastenhouw et al., 2010). Although these findings do not reveal much detail about the role of histone modifications in regulation of global transcriptional activity, a

more recent study implies a more complex role for preMBT chromatin architecture in regulating transcriptional competency during the MZT, as discussed later in this chapter (Blythe et al., 2010).

Part II: Transition to zygotic control

Maternal mRNA destabilization

Degradation of maternal mRNAs occurs during the MZT in *Drosophila*, zebrafish, C. elegans, mice, and *Xenopus*, and is usually mediated by destabilization of mRNAs via deadenylation mechanisms (Voeltz and Steitz, 1998; Baugh et al., 2003; Hamatani et al., 2004; Mathavan et al., 2005; De Renzis et al., 2007). Although deadenylation of many transcripts occurs upon fertilization, decay of these transcripts is not manifested until just prior to the MBT, with the exception of *Xenopus* where maternal mRNA degradation is usually seen following zygotic genome activation (Duval et al., 1990; Audic et al., 1997; Audic et al., 2001; Audic et al., 2002; Lund et al., 2009). In *Xenopus*, deadenylation is typically regulated by AU-rich cis-elements within maternal mRNAs, and the Embryonic Deadenylation Element Binding Protein (EDEN-BP), which work together to de-adenylate many maternal transcripts (Paillard et al., 1998; Voeltz and Steitz, 1998). Although initial reports suggested that mRNA degradation is not dependent on gene expression (Duval et al., 1990), more recent reports show that maternal mRNA degradation does require new transcription (Audic et al., 2001; Audic et al., 2002), which may in part explain why maternal transcript degradation is seen only following the MBT in *Xenopus*.

A recent study implicates the zygotically expressed miRNA miR-427 in the deadenylation of maternal mRNAs following the MBT in *Xenopus* (Lund et al., 2009). Additionally, zebrafish and *Drosophila* each have a zygotically expressed orthologue of miR-427: miR-430 and miR-309, respectively. These microRNAs are required for the degradation of hundreds of maternal transcripts in the early fish and fly embryo (Giraldez et al., 2006; Bushati et al., 2008). While zygotic transcription might play a role in

mediating destruction of deadenylated maternal mRNAs during the MBT, it does not explain the phenomenon entirely, since destruction of many mRNAs occurs before zygotic transcription begins; in *Xenopus* and *Drosophila*, many studies have suggested that this feature of the MBT might be controlled by a timing mechanism activated at fertilization, and not by number of cell cleavages or the nuclear-to-cytoplasmic ratio per se (Howe and Newport, 1996; Bashirullah et al., 1999; Tadros et al., 2003).

In *Drosophila*, the RNA binding protein Smaug (SMG) is a key factor in mediating events at the maternal to zygotic transition including maternal transcript destabilization. SMG binds to SMG-recognition elements within maternal transcripts and recruits the CCR4/POP2/NOT-deadenylase complex (Semotok et al., 2005; Semotok et al., 2008). SMG is also essential for restructuring of the cell cycle, activation of DNA replication checkpoints, cellularization, and robust zygotic gene activation at the MBT.

The role of maternal mRNA destruction during the maternal-to-zygotic transition is not entirely clear. One obvious possibility would be a mechanism to ensure the correct levels of a given transcript are present when it begins to accumulate zygotically – this would necessitate any maternal contribution of that RNA is cleared. Another attractive possibility is that ubiquitously-expressed maternal RNAs need to be removed so that the embryo can generate a more restricted pattern of gene expression when zygotic transcription begins. Some evidence shows that within the subset of zygotic genes that replace ubiquitously expressed maternal counterparts, genes with refined patterns of zygotic expression are over-represented (De Renzis et al., 2007). Alternatively, the clearance of maternal RNAs could serve as a developmental cue to direct events at the MBT; for example, the reduction of maternal cell cycle regulators has been correlated with the changes in cell-cycle dynamics as the MBT approaches, and is discussed in more detail later in this chapter (Audic et al., 2001; Audic et al., 2002; Lund et al., 2009).

Another example is seen in the study of the *Drosophila* RNA-binding protein SMG. One of the maternal mRNAs targeted for degradation at the MBT by SMG is the maternal transcriptional repressor *tramtrack*, which suggests that SMG might mediate activation of zygotic gene expression through the removal of a transcriptional repressor (Benoit et al., 2009).

Cell cycle and motility changes at the MBT

As discussed earlier in this chapter, cells of the early *Xenopus* embryo cycle through rapid S and M phases with no intervening G phases, with each cycle lasting about 20 to 30 minutes depending on the clutch and temperature. After the 12th embryonic cleavage division when the embryo has reached the 4000-cell stage, the cell cycle begins to slow down; first the S and the M phases slow followed by introduction of a G1 and a G2 phase, resulting in a 13th cell cycle whose duration is more than twice that of the previous (Graham and Morgan, 1966; Newport and Kirschner, 1982a; Frederick and Andrews, 1994). It is also at this time that the cell cycles between different blastomeres of the embryo become highly asynchronous in a size-dependent manner (vegetal pole cells having a larger radius and thus being more asynchronous with one another than animal pole cells), and cell cycle checkpoints are first introduced (Newport and Kirschner, 1982a; Shimuta et al., 2002; Conn et al., 2004; Iwao et al., 2005). The change in cell cycle length and the addition of G phases associated with the MBT is controlled by a series of events that occur around this time. Degradation of CyclinE and Cdc6, both required for DNA synthesis, is involved in the extension of S phase at MBT; intriguingly, this activity does not seem to be dependent on zygotic gene activation or nuclear-to-cytoplasmic ratio (Howe and Newport, 1996; Hartley et al., 1997; Tikhmyanova and Coleman, 2003). M phase extension and the introduction of G phases

appear to be mediated by a transient increase in Chk1 activity at the MBT leading to degradation of Cdc25, a phosphotase required for the activation of Cdc2 and progression to M phase, as well as the activation of Wee1 which is required to further inactivate Cdc2 (Ferrell et al., 1991; Kim et al., 1999; Lee, J. et al., 2001; Okamoto et al., 2002; Shimuta et al., 2002). Although introduction of cell cycle checkpoints may be a consequence of cell cycle lengthening and addition of G phases at the MBT, several studies have shown that cell-cycle checkpoints are acquired once the cells of them embryo reach a critical nuclear-to-cytoplasmic ratio, a recurring theme for control of many of the events that occur at the MBT (Conn et al., 2004; Peng et al., 2008).

In the syncytial blastoderm embryo of *Drosophila*, the slowing of the nuclear cycle and rearrangement to include G phases appears to be under similar control to that of *Xenopus*, although unlike *Xenopus*, it occurs over multiple (3-4) cleavage divisions instead of one, culminating at the 14^{th} nuclear cycle when cellularization occurs (Pritchard and Schubiger, 1996; Ji et al., 2004). Phosphorylation levels of the *Drosophila* homologue of Cdc2 (Cdk1) remain low throughout cleavages, and then reach their highest level at cycle 14 (MBT), in a Wee1-dependant manner (Edgar et al., 1994; Campbell et al., 1995; Stumpff et al., 2004). Also similar to *Xenopus*, *Drosophila* Cdc25 (String) maternal levels remain high during cleavage stages, then gradually decline at the beginning of the transition (cycle 10) as maternal mRNAs are degraded, and are at their lowest levels by cycle 14 (Edgar and Datar, 1996; McCleland et al., 2009). It is also during this transition in *Drosophila*, between cleavages 10 to 14, that cell cycle checkpoints become active, as the cell cycle slows and G phases begin to reappear in the embryo (Sibon et al., 1997). Similarly, in zebrafish, cell cycle lengthening and checkpoint control are mediated by a coordination of the activities of Cdc25 and Cdk1,

and occur at the same time as large-scale genome activation, after the 10th cleavage division (Dalle Nogare et al., 2009).

In *Xenopus*, prior to the MBT mitotic cells are not motile; however after the 12th cleavage division, pseudopodal processes begin to form on many cells, and they begin to translocate their position within the embryo, such that within 90 minutes of cleavage 12, 85% of small-diameter (<0.1mm) are motile (Satoh et al., 1976; Newport and Kirschner, 1982a). The transition from non-motile to motile cells most likely occurs as a consequence of lengthening of the cell cycle. It is largely thought that the rearrangement of the microtubule network and the remodeling of the actin cytoskeleton required for DNA segregation and cytokinesis, respectively, is incompatible with the cytoskeletal arrangements necessary for cell migration (Duncan and Su, 2004). Indeed, evidence in both *Xenopus* and *Drosophila* demonstrates that cells manipulated to continue cleaving rapidly after the MBT are inhibited from cell motility and gastrulation movements, despite the fact that other milestones of the MBT such as maternal mRNA degradation and zygotic transcription appear normal, suggesting a more complex regulatory mechanism between cell cycle control and other events of the MBT (Grosshans and Wieschaus, 2000; Mata et al., 2000; Murakami et al., 2004).

Large scale genome activation

Large-scale activation of the zygotic genome can be considered one of the last steps in the maternal-to-zygotic transition for many organisms. For *Xenopus*, this is observed by radio-labeled uridine incorporation into newly synthesized RNA after the 12th cleavage division, when the embryo has reached the 4000-cell stage (Newport and Kirschner, 1982a); in zebrafish, this occurs at the 10th cleavage division when the embryo has reached 1000 cells (Kane and Kimmel, 1993). In *Drosophila*, large-scale

zygotic genome activation occurs during the 14th nuclear cycle when the cell cycle slows dramatically and cellularization of nuclei occurs (Edgar and Schubiger, 1986).

The simultaneous activation of transcription throughout the embryo at the MBT suggests the presence of a signal that coordinates this response. As discussed previously, many studies show that the embryo responds to a change in the nuclear-to-cytoplasmic ratio at the MBT, which led to the hypothesis that a transcriptional repressor is titrated out with successive nuclear divisions (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). Several factors have presented themselves as potential candidates for the titratable repressive factor in *Xenopus*. As discussed above, the maternal regulators of repressive DNA methylation, Kaiso and Dnmt1 both show gene repression activity before the MBT and depletion of these factors leads to premature expression of many MBT genes (Ruzov et al., 2004; Dunican, D. S. et al., 2008). Additionally, *tramtrack* seems to play an active role in repressing some genes in preMBT *Drosophila* embryos (Pritchard and Schubiger, 1996). Clearly these factors are not responsible for repression of the entire genome, as their depletion leads to the preMBT activation of only a handful of genes suggesting other repressive mechanisms are in place.

It seems that the combination of a limitation of transcriptional activators, a repressive chromatin environment and the presence of multiple transcriptional repressors are the main contributors to repression of transcription in the early embryo. At the MBT, a combination of the changing nuclear-to-cytoplasmic ratio and destruction of maternal mRNAs contributes to the depletion of repressive transcription factors, modification in chromatin structure, change in cell cycle dynamics, and regulation of transcriptional activators, all of which work together to allow a more transcriptionally permissive environment.

Recent studies in *Drosophila* suggest a more complicated timing mechanism coordinates the timing of the events at the MBT, including onset of large-scale gene expression. By manipulating DNA content in the developing embryo, Lu and colleagues found that the time of activation of only a fraction of genes is controlled by the nuclear-to-cytoplasmic ratio (Lu et al., 2009). The majority of the genes activated at the ZGA, as well as the degradation of most maternal mRNAs are controlled by an absolute stage or time sensing mechanism, and the onset of expression of these genes in not changed by a change in the nuclear-to-cytoplasmic ratio. However changes in duration of the cell cycle at the MBT do seem to be under the control of nuclear-to-cytoplasmic ratio; it will be interesting to investigate the various mechanisms underlying the onset of different events at the MBT, and if these mechanisms share any components that might contribute to the synchrony of these events during early development.

Part III: PreMBT transcription

Several groups have reported that new RNA synthesis is undetectable before the MBT (Bachvarova and Davidson, 1966; Woodland and Gurdon, 1969; Newport and Kirschner, 1982a). However evidence for transcription of zygotic genes before the MBT has emerged over the last several decades (Edgar and Schubiger, 1986; Kimelman et al., 1987; Nakakura et al., 1987; Shiokawa et al., 1989; Yasuda and Schubiger, 1992; Yang, Jing et al., 2002), and a handful of genes have now been confirmed as zygotically active prior to the MBT in a number of organisms (Yang, Jing et al., 2002; Leung et al., 2003a; Mathavan et al., 2005; ten Bosch et al., 2006; De Renzis et al., 2007; Rosa et al., 2009). However, aside from some interesting work in *Drosophila* (described below), the regulation of preMBT transcription, and more importantly, its developmental role, remains a poorly understood phenomenon. In this work I characterize the contribution of a particular maternal factor to preMBT transcription in the *Xenopus*, and identify a critical developmental role for preMBT gene expression.

preMBT transcription revealed

When Kimelman and colleagues repeated radioactive uridine incorporation experiments similar to those performed by Newport and Kirschner (1982) and Bachvarova and Davidson (1966), their initial findings were quite similar: new transcript accumulation only occurs after the 12th cleavage division (Kimelman et al., 1987). However, when they exposed the same radiograms to film for a much greater length of time, they were able to resolve that new transcripts have accumulated, albeit in very low levels, by the 128-cell stage (after the 6th cleavage division). Several other groups also noted synthesis of both high and low molecular weight RNAs before the MBT in both

Xenopus and *Drosophila* (Edgar and Schubiger, 1986; Nakakura et al., 1987). Later, careful in-situ hybridization, RT-PCR, qRT-PCR, and micro-array analysis in fly, fish and frog revealed the identity of a handful of specific RNAs transcribed several cell cycles prior to the MBT (Pritchard and Schubiger, 1996; Yang, J. et al., 2002; Leung et al., 2003a; Mathavan et al., 2005; Lund et al., 2009). It has become clear that as emerging technologies allow for progressively more sensitive methods of detection of low-levels of gene products, more factors actively transcribed before the MBT will be identified.

preMBT transcription in *Xenopus laevis*

Transcription of the TGFβ ligands Nodal-related 5 and Nodal-related 6 (*xnr5* and *xnr6*) before the MBT was first reported by Yang and colleagues, after a careful study of the temporal function of Wnt signaling (Yang, J. et al., 2002). The Wnt/β-catenin signaling pathway, is an integral part of early axis specification, as will be discussed in Part IV of this Introduction. Interestingly, activity of the β-catenin/TCF transcriptional apparatus is required during early cleavage stages for later dorsal development. β-catenin has been observed to translocate to the nucleus, indicative of active Wnt signaling, as early as the 16-cell stage (Larabell et al., 1997). Additionally, the effects of rescue of UV-ventralized embryos by activation of Wnt signaling with lithium chloride are maximally achieved when lithium is added to the medium at the 32 to 64-cell stage, and declines after that period, suggesting that Wnt signaling is required during this early period to specify dorsal cell fates (Kao et al., 1986). Additionally, inhibition of Wnt signaling with anti-sense morpholino oligonucleotides (MO) produces a highly ventralized phenotype when MO is injected by the 4-cell stage; however if β-catenin MO is injected at the 8 or 16-cell stage, the phenotype is much less severe (embryos

develop full dorsal structures), suggesting an early role for Wnt signaling in pattering the embryo (Heasman et al., 2000).

To explain these findings and to establish when Wnt signaling is required for dorsal development, Yang and colleagues used a hormone-inducible dominant-negative TCF construct to temporally inhibit Wnt signaling in dorsal embryonic cells (Yang, J. et al., 2002). Inhibition of Wnt signaling at the 4 cell stage results in ventralization whereas inhibition at 32 cells or later does not have as severe an effect, suggesting that the embryo responds to Wnt signaling in a short window of time before the 32 cell stage. They proposed that inhibition of Wnt signaling after this early time is not as effective in producing a ventralized embryo because the embryonic response has already been established by the 32 cell stage. Interestingly, they were also able to extend this window to later stages when they transiently inhibited transcription with actinomycin D, suggesting that the embryonic response to Wnt signaling in this early window was transcriptionally mediated. When they examined RNA that had been labeled with P32-UTP in intact, cleaving embryos, they showed accumulation of high molecular weight, polyadenylated RNAs before the MBT. They then analyzed the expression of several targets of Wnt/β-catenin signaling by RT-PCR and found that new *xnr5* and *xnr6* transcripts are detectable as early as the 256-cell stage; they further showed that their levels increase during preMBT development, and that their preMBT expression requires RNAP II and β-catenin. They speculated that Wnt-mediated preMBT transcription was necessary for future dorsal development in the embryo.

However it has been difficult to address the function of preMBT transcription in the *Xenopus* embryo beyond this study, as most chemical inhibitors of transcription are either not reversible (the RNAP II inhibitor α-amanitin), or have non-specific effects on cells, (the DNA intercalation agent and transcriptional inhibitor actinomycin D).

Additionally, it has been challenging to demonstrate the function of *xnr5* and *xnr6* in the embryo at any stage; antisense morpholinos designed against these transcripts had been largely ineffective, most likely due to the duplication of *xnr5* in the *Xenopus* genome, resulting in 15 variant copies (Takahashi et al., 2006). However, Luxardi and colleagues recently identified effective morpholinos against *xnr5* and *xnr6* and showed that simultaneous loss of function of *xnr5* and *xnr6* reduces overall Nodal signaling in the embryo and leads to a loss of early dorsal mesodermal markers; although this demonstrates that *xnr5* and *xnr6* are important for dorsal development in the embryo, it does not establish that their preMBT function per se is required for dorsal development. (The results of this study and the contribution of Nodal signaling to development will be discussed in more detail in Part IV of this chapter.)

Interestingly, the *Xenopus* microRNA miR-427 has recently been shown to be expressed as early as one hour before the MBT (Lund et al., 2009). As discussed earlier in this chapter, miR-427 was shown to be an important regulator of maternal mRNA degradation and subsequent regulation of cyclins involved in remodeling of the cell cycle at MBT. Intriguingly, this microRNA was also shown recently to be involved in regulation of Nodal signaling in the early embryo, including regulation of *xnr5* and *xnr6* (Rosa et al., 2009), although it remains to be seen if the effects of Nodal regulation by miR-427 occur before the preMBT.

preMBT transcription in *Drosophila*

Large scale gene activation in *Drosophila* begins during the 14th cell cycle when cellularization and dramatic cell cycle slowing occurs (Lamb and Laird, 1976; McKnight and Miller, 1976; Anderson and Lengyel, 1979; Foe and Alberts, 1983; Edgar and Schubiger, 1986). However, the transition from maternal to zygotic control in *Drosophila*

is more gradual then in other organisms discussed here; nuclear cycles begin to slow for the first time during the 10th nuclear division, nuclei begin to migrate to the cortex of the blastoderm and nuclear cycles 11, 12 and 13 gradually become slower (Pritchard and Schubiger, 1996). Although the bulk of gene expression is considered to occur during the 14th nuclear cycle, many genes begin to be expressed during cycles 10 through 13, when cycle slowing occurs (Ingham et al., 1985; St Johnston and Gelbart, 1987; Eldon and Pirrotta, 1991; Ibnsouda et al., 1993; Schejter and Wieschaus, 1993; Barbash and Cline, 1995; Pritchard and Schubiger, 1996; De Renzis et al., 2007); intriguingly, even before slowing beings, expression of some genes can be seen as early as nuclear cycle 8 (Erickson and Cline, 1993; Pritchard and Schubiger, 1996; De Renzis et al., 2007; Porcher et al., 2010). Although no specific studies have addressed if the preMBT expression of these genes is important for later development, some of these genes are known to be required for cellularization during the 14th cell cycle (Erickson and Cline, 1993; Ibnsouda et al., 1993; Pritchard and Schubiger, 1996), while others are known to be involved in sex-determination which also occurs by the 14th cell cycle (Erickson and Cline, 1993; Barbash and Cline, 1995). Additionally, the early activation of many of these genes is under the control of specific regulatory sequences and maternal transcriptional activators (ten Bosch et al., 2006; Harrison et al., 2010); this will be discussed in more detail in the next section.

preMBT transcription in zebrafish

The Wnt-target gene bozozok is expressed as early as the 500-cell stage in zebrafish embryos, at least 1 cell cycle prior to the MBT (Leung et al., 2003a). Although bozozok is important for specifying axial mesoderm, whether its preMBT expression is important for development has not been addressed (Leung et al., 2003b). Recently, a

microarray comparing gene expression in different stages of zebrafish embryos identified up to 125 zygotic genes that are potentially expressed prior to the MBT (Mathavan et al., 2005). Although preMBT expression of a few of these genes was validated by in situ, it remains unclear if the majority of the reported genes represents new transcription or changes in polyadenylation of maternal mRNAs.

Maternal transcription factors regulate preMBT transcription

Although postMBT activation of *xnr5* and *xnr6* requires input from both β-catenin and the T-box transcription factor VegT (Rex et al., 2002; Hilton et al., 2003), the contribution of VegT to preMBT activation of *xnr5* and *xnr6* has not been studied; in this work, I determine that the preMBT expression of *xnr5* and *xnr6* as well as another group of genes important for mesendodermal development requires the maternal transcription factor VegT.

In *Drosophila*, many of the genes expressed before the MBT contain a conserved heptanucleotide repeat (CAGGTAG) in their promoter regions (Erickson and Cline, 1998; ten Bosch et al., 2006). These sites, termed "TAGteam sites," are crucial for expression before the MBT: adding or removing these sites to the regulatory region of a gene advanced or delayed its expression, respectively (ten Bosch et al., 2006). The maternal zinc-finger transcription factor Zelda binds to these sites and is required for activation of genes before the MBT in *Drosophila* (Liang et al., 2008). A maternal competitive inhibitor of transcription, Grainyhead, also binds to TAGteam-like sites before the MBT, leading to the model that Grainyhead binds to and represses preMBT genes early in development; as Zelda levels increase (through either new translation or transcription), Grainyhead is gradually outcompeted for binding to TAGteam sites by Zelda, which begins to activate

transcription midway through cleavage stages (Harrison et al., 2010). No similar regulatory sequence has been identified in *Xenopus*.

The role of preMBT gene expression in the embryo

Although regulated gene expression before the MBT is a conserved phenomenon in many metazoans, a developmental role of specific transcripts has yet to be defined. In *Drosophila*, several genes expressed before the MBT are important for sex-determination and cellularization, both events that occur at the MBT (Barbash and Cline, 1995; Pritchard and Schubiger, 1996; Erickson and Cline, 1998). Interestingly, mutation of the preMBT transcription factor Zelda does lead to defects in cellularization, but whether this is due to a loss of a specific preMBT transcript has not been addressed (Liang et al., 2008). The Nodal genes *xnr5* and *xnr6* are expressed well before the MBT in *Xenopus*. Although Nodal signaling is essential for mesoderm formation and patterning in the embryo, the contribution of specific Nodals to mesoderm formation has only recently been addressed (Luxardi et al., 2010). Importantly, the significance of zygotic Nodal signaling before the MBT to embryonic development has not been addressed. In this work, I demonstrate that the RNAP II mediated transcription of *xnr5* and *xnr6* before the MBT is necessary to activate early Nodal signaling in the embryo, and that activation of Nodal signaling before the MBT is necessary for normal development of the embryo.

Part IV: Early pattern formation in the frog

In this thesis, I show that preMBT activation of gene expression is directed by a maternal regulator of endodermal and mesodermal (mesendodermal) pattern formation, VegT. Additionally, I show that two zygotic genes expressed before the MBT in frog, *xnr5* and *xnr6*, play a crucial role in mesoderm formation in the early embryo. This section will review what is known about the maternal and zygotic contributions to early pattern formation in the frog, focusing on the maternal T-box transcription factor VegT and the early zygotic Nodal signaling pathway.

Mesoderm induction in the frog

All tissues of the adult frog arise from three primary germ layers, endoderm, ectoderm and mesoderm. Components that specify the endoderm and the ectoderm are associated with the vegetal and animal poles of the embryo, respectively. Indeed, when the animal pole of the early embryo is excised and cultured individually it produces ectodermal tissue, primarily epidermis, and when the vegetal mass is excised and cultured it develops into endoderm. However, when the excised animal pole is cultured in contact with the vegetal mass, the animal pole tissue is induced to form mesoderm (Nieuwkoop, 1969)(Smith, 1989). This suggests that a secreted factor in the vegetal mass can induce animal pole tissue to become mesoderm. Subsequent studies characterizing the molecular nature of this mesoderm-inducing signal suggest that it is the result of activity from the TGFβ signaling family. Loss of function studies further identified Nodal signaling as the major mesoderm-inducing component in the early embryo (Hemmati-Brivanlou and Melton, 1992; New et al., 1997; Agius et al., 2000). The contribution of Nodal signaling to mesoderm development will be discussed in detail later.

Other signaling pathways contributing to mesoderm development include the BMP and FGF signaling pathways. The BMP signaling pathway, like Nodal, is a member of the TGFβ signaling superfamily. However BMPs are responsible exclusively for the induction of ventral mesoderm in *Xenopus*, and loss of function leads to an expansion of dorsal mesoderm at the expense of ventral mesoderm (Graff et al., 1994; Suzuki et al., 1994). Although BMP ligands are maternally expressed in the frog (Hemmati-Brivanlou and Thomsen, 1995), they do not signal until after the MBT (Schohl and Fagotto, 2002). BMP is initially seen throughout the mesoderm at stage 9 and becomes restricted to the ventral mesoderm as development continues, due to the effects of the dorsal organizer, several targets of which are direct BMP antagonists (Schohl and Fagotto, 2002; De Robertis, 2009). Loss of function experiments show that FGF signaling is also important for ventral mesoderm in the developing embryo (Amaya et al., 1991; Amaya et al., 1993). However, studies indicate that FGF serves as a maintenance factor for mesoderm development, and not as a mesoderm inducer (Fletcher and Harland, 2008).

Maternal control of pattern formation in the frog

One of the first steps in embryogenesis involves the establishment of the primary body axes and generation of the primary germ layers. In *Xenopus*, animal-vegetal polarity is established during oogenesis, a process that involves the localization of yolk platelets and crucial maternal mRNAs and proteins to the vegetal pole of the embryo where much of the future endoderm will be generated, as well as the localization of maternal mRNAs and proteins to the animal pole where the future ectoderm will form. Patterning of the axes is further refined by maternal factors after fertilization, when the site of sperm-entry dictates positioning of the future dorsal-ventral axis of the embryo. The molecular pathways downstream of these initial patterning cues are under tight

spacio-temporal regulation from both maternal and zygotic patterning cues. Here I will discuss the maternal contribution to patterning of the mesoderm and the dorsal-ventral axis of the embryo, with a particular focus on those pathways involved in preMBT gene expression: Wnt and VegT.

Maternal Wnt signaling

Fertilization of the *Xenopus* egg triggers a microtubule-driven reorganization of the cytoplasm through a process known as cortical rotation where the outer-most or cortical cytoplasm rotates with respect to the inner-most cytoplasm during the first cell cycle. The result of this process is that a maternally localized dorsalizing activity is translocated to the future dorsal side of the vegetal pole, typically diagonally opposite from the point of sperm entry (Vincent and Gerhart, 1987). A proposed candidate for this dorsalizing activity is maternal Wnt11, whose mRNA and protein can be observed in the dorsal-vegetal zone of the cleavage-stage embryo (Tao et al., 2005). This localized Wnt activity results in stabilization of the downstream effector β-catenin, allowing for β-catenin accumulation and translocation into the nucleus where it binds to Lef/Tcf transcription factors and activates target genes. Early dorsal Wnt targets include the organizer genes and the ventral BMP/zygotic Wnt antagonists *siamois*, *twin*, *goosecoid*, *xnr3*, *chordin*, *cerberus* and *noggin* (Cho et al., 1991; Smith and Harland, 1992; Lemaire et al., 1995; Smith et al., 1995; Bouwmeester et al., 1996; Piccolo et al., 1996; Yao and Kessler, 2001); expression of these genes in the dorsal marginal zone in response to early Wnt signaling is crucial for the establishment of the embryonic organizer. In the absence of Wnt ligand on the ventral side of the early embryo, β-catenin is targeted for degradation by GSK-3 kinase activity; Lef/Tcf is not bound by β-catenin, and acts instead as a transcriptional repressor for Wnt target genes during this early time.

The lymphoid enhancer factor/T-cell factor (Lef/Tcf) family of DNA transcription factors is also present maternally, and is ubiquitously distributed throughout the embryo (Molenaar et al., 1996; Roel et al., 2003). These DNA binding proteins are important transducers of the Wnt signaling pathway, allowing transcriptional activation of target genes when bound to β-catenin. In the absence of β-catenin, Lef/Tcf is bound by CtBP and/or groucho, both factors that recruit transcriptionally repressive HDAC activity to promoters (Chinnadurai, 2002; Houston et al., 2002; Daniels and Weis, 2005). Loss of function studies show that depletion of Tcf3 leads to an upregulation of Wnt target genes, highlighting the role of Tcf3 as a repressor, and suggesting that other Tcf genes can act redundantly in serving as a transcriptional activating partner for β-catenin (Houston et al., 2002). The activating and repressive roles for Tcf demonstrate that it is a dynamic maternal transcription factor, serving many roles in patterning the early embryo.

A recent study in our laboratory found that preMBT activity of the Wnt/β-catenin pathway is required not just for transcription of organizer genes and *xnr5* and *xnr6*, but also for the establishment of permissive chromatin architecture at genes expressed after the MBT (Blythe et al., 2010). As mentioned in Part III of this chapter, many lines of evidence suggest that the Wnt/β-catenin pathway must be active during very early cleavage stages to ensure dorsal specification. However, with the exception of *xnr5* and *xnr6*, none of the maternal Wnt targets is expressed before to the MBT. Recent work found that β-catenin is crucial for recruiting the histone H3 argentine 8 methyltransferase Prmt2 to promoters of organizer-specific genes, thus establishing poised chromatin architecture at dorsal promoters during the preMBT period so that they are primed for activation after the MBT (Blythe et al., 2010). Through loss-of-function studies and the use of a Prmt2-Lef fusion construct, they also showed that recruitment of Prmt2 to early Wnt-target promoters is necessary and sufficient for dorsal axis formation. These

findings demonstrate a new role for dorsally localized β-catenin and preMBT Wnt signaling in poising permissive chromatin architecture at early postMBT genes for later expression, ensuring the establishment of the dorsal axis.

VegT

VegT is a maternal T-box transcription factor that is critical for formation and patterning of the mesoderm and the endoderm. VegT mRNA is localized to a broad region in the vegetal pole during oogenesis, and anchored by a localization sequence in its 3' UTR (Zhang and King, 1996; Zhao et al., 2001). Whole mount immunostaining reveals that maternal VegT protein is localized to the nucleus of vegetal cells by the 32 cell stage and perhaps earlier (Stennard et al., 1999). VegT activates zygotic determinants of endoderm and mesoderm, exclusively in the vegetal region of the embryo. Targets include endoderm-specific transcription factors including *bix4*, *sox17*a, *mixer* and GATA factors (Xanthos et al., 2001; Kofron, M. et al., 2004). Along with dorsally-stabilized β-catenin, VegT also activates expression of the BMP/zygotic-Wnt antagonists *chordin*, *cerberus*, *noggin* and *dkk* in the dorsal-vegetal domain of the postMBT embryo, an important event for the establishment and maintenance of the embryonic organizer (Xanthos et al., 2001; Xanthos et al., 2002). Finally, VegT activates expression of Nodal ligands in the vegetal portion of the embryo, including *xnr1*, *xnr2*, *xnr4*, *xnr5*, *xnr6* and *derrière*, critical signaling molecules for the induction of mesoderm and for gastrulation movements in the embryo (Zhang et al., 1998; Kofron et al., 1999; Luxardi et al., 2010). Particularly important for this work, is the activation of zygotic expression of *derrière*, *xnr5* and *xnr6* by VegT.

Studies depleting maternal VegT mRNA have shown that VegT is essential for the establishment of the primary germ layers (Zhang et al., 1998; Kofron et al., 1999).

When maternal VegT is depleted, embryos lose markers of endoderm and mesoderm at the expense of an expanded ectodermal territory. By tailbud stage, the embryonic axes have not formed and the embryo appears to be a radialized mass of ectodermal tissue. Additionally, while control vegetal explants will induce mesoderm in animal cap tissue when recombined, VegT deficient vegetal explants do not induce mesoderm, suggesting that mesoderm is specified by secreted targets of VegT.

Another, less characterized role for VegT is in the localization of other RNAs during oogenesis. When VegT is depleted from oocytes using antisense phosphorothioate oligonucleotides, which target VegT mRNA for degradation, several other vegetally localized mRNAs become delocalized, but this is not observed with translation blocking antisense morpholino oligonucleotides targeted against VegT (Heasman et al., 2001). Further analysis revealed that depletion of VegT mRNA disrupts the cytokeratin network in the vegetal cortex, which perhaps explains the failure for other vegetally localized mRNAs such as Wnt11 and Vg1 to localize in VegT-depleted embryos (Kloc et al., 2007).

VegT mRNA is localized to the vegetal pole of the oocyte during oogenesis via a sequence in the 3' UTR; this process makes VegT mRNA only available for translation to cells in the vegetal region of the embryo, and is a key feature of spatial regulation of VegT activity by the embryo. Anchoring of VegT mRNA to the vegetal pole is in part due to the action of the vegetally localized proline-rich protein (Prrp), which binds to the 3' UTR of VegT and other vegetally localized mRNAs, as well as components of the cytoskeleton (Zhao et al., 2001). Stability of VegT can also be regulated by the RNA-binding protein Seb4R, which binds to the 3' UTR of VegT and enhances translation and stability of the message (Souopgui et al., 2008). Although Seb4R is distributed throughout the embryo and thus does not contribute to regional stability of VegT mRNA,

it is nonetheless important for VegT function, since depletion of Seb4R disrupts germ layer formation. VegT activity is also negatively regulated in the animal pole by animally-localized POU-V factors including Oct25 and Oct60. It is thought that these Oct proteins can bind specifically to VegT and Tcf at dorsal target promoters, where they inhibit expression in the animal pole, ensuring that dorsal-vegetal gene expression is restricted to the dorsal-vegetal and marginal regions of the embryo (Cao et al., 2007).

Maternal components of the Nodal/Activin signaling pathway

The Nodals/Activin-like ligands are members of the TGFβ superfamily of signaling molecules and are essential for the specification and patterning of mesoderm in the blastula, as well as cell movements during gastrulation. Nodal/Activin-like ligands transduce their signals through type I and type II serine-threonine kinase receptors, resulting in phosphorylation of Smad2 or Smad3; phosphorylated Smad2/3 then associates with the co-Smad Smad4, translocates to the nucleus, and associates with transcriptional cofactors to activate target gene expression. In the frog, mesoderm induction occurs largely as a result of zygotically expressed Nodal-related ligands (Xnrs), however, some components of the Nodal signaling pathway are maternally expressed.

The TGFβ ligand Activin is a potent inducer of mesoderm and was initially identified as a secreted mesoderm-inducing activity in *Xenopus* cell lines (Smith et al., 1990; Sokol et al., 1990). Activin can potentially act as a mesoderm-patterning morphogen: high levels of activin induce dorsal mesoderm, low levels induce ventral mesoderm, and intermediate levels produce lateral mesoderm (Gurdon et al., 1994; Gurdon et al., 1997). Activin is maternally expressed, and although it is an attractive candidate for endogenous mesoderm induction, inhibition of activin activity in the embryo

shows that endogenous activin plays only a minor role in development of endogenous mesoderm during late gastrula stages (Piepenburg et al., 2004).

Vg1 is a maternal TGFβ whose mRNA is vegetally localized in the oocyte in a manner similar to VegT mRNA. Because of its localization to prospective endoderm and the finding that TGFβ molecules can induce mesoderm in vitro, this molecule was suggested to be the mesoderm-inducing signal proposed by Nieuwkoop and colleagues (Kimelman and Kirschner, 1987; Weeks and Melton, 1987). However further analysis demonstrated that Vg1 protein is not processed to its bioactive form in vivo, and is not secreted efficiently, making the role of Vg1 in patterning the early embryo difficult to interpret (Dale et al., 1989; Tannahill and Melton, 1989; Dale et al., 1993). More recent data exploring loss of function of Vg1 suggests that Vg1 indeed plays a role in Nodal signaling and mesoderm formation (Joseph and Melton, 1998; Birsoy et al., 2006). This led to the discovery of a second pseudoallele of Vg1, named Vg1(S), that is processed more efficiently in embryos, has mesoderm-inducing activity, and rescues Vg1-depleted embryos (Birsoy et al., 2006). Depletion of Vg1 causes delayed blastopore formation, anterior defects and a loss of some P-Smad2 signal in the embryo. The authors note however, that Vg1(S) is not processed to its mature form as efficiently as other Nodals such as *xnr5*, and that zygotic Nodals are much more efficient mesoderm-inducers, further illustrating that although Vg1 may play a role in early patterning, other Nodal related genes are crucial for the induction of mesoderm (Birsoy et al., 2005; Birsoy et al., 2006).

One of the major players in Nodal signal transduction is the forkhead DNA binding protein FoxH1, which serves as a transcriptional partner for activated Smad2/Smad4 complexes. FoxH1 is required for survival in mouse embryos, and deficiency results in embryos that fail to form the node, prechordal mesoderm, notochord

and definitive endoderm (Hoodless et al., 2001; Yamamoto et al., 2001). Interestingly, FoxH1 is exclusively maternally provided in the *Xenopus* embryo, and is essential for the development of head features, although loss of FoxH1 function does not preclude the embryo from responding to Nodal signals, suggesting an alternative Smad-interacting transcription factor transduces Nodal signals in the absence of FoxH1 (Kofron, Matt et al., 2004). The zygotic transcription factor *Mixer* has been proposed to fill such a role (Germain et al., 2000). FoxH1 is also necessary for the zygotic activation of Nodal targets such as *chordin*, *xnr3* and *goosecoid*; interestingly, evidence from these experiments suggests that FoxH1 is also important for the ventral repression of *xnr5* and *xnr6* in the absence of Nodal signal, demonstrating that FoxH1 can function as a repressor in the absence of signal and as an activator in the presence of signal.

Additional maternal components of Nodal signaling include the tumor suppressor p53, and the proprotein convertase PACE4. Recent work in *Xenopus* shows that p53 associates with Smad2/3 in a Nodal ligand-dependent manner, and is important for specification of mesoderm (Cordenonsi et al., 2003; Takebayashi-Suzuki et al., 2003). PACE4 mRNA is vegetally localized in the *Xenopus* embryo and PACE4 protein is important to convert TGFβ ligands to their mature, bioactive form. PACE4 is required for the processing of some but not all Nodal ligands, therefore although embryos lacking PACE4 have reduced mesoderm and Smad2 phosphorylation, limited mesoderm specification still occurs (Birsoy et al., 2005).

Zygotic control of early pattern formation

The embryonic organizer

Early experiments by Hilde Mangold and Hans Spemann demonstrated that the dorsal blastopore lip (DBP) of the gastrulating embryo has powerful inductive properties (Spemann and Mangold, 1924). When the DBP is excised from an embryo and transplanted to a new embryo, the host embryo develops a twinned, secondary axis, complete with all the features of a whole embryo. Lineage tracing experiments revealed that this secondary axis is created mainly from cells of the host embryo, demonstrating the influential instructional nature of the DBP, an area of the embryo now referred to as the Spemann organizer. Molecularly, we now know the organizer is initially specified by maternal β-catenin signaling in the dorsal-vegetal region of the embryo as explained in the previous section, with some contribution from Nodal signaling (Agius et al., 2000; De Robertis, 2009). Although the establishment of the organizer is controlled maternally, the molecular factors characteristic of the organizer are zygotically expressed genes. These organizer genes, including *noggin*, *chordin*, *xnr3*, *siamois*, twin, *cerberus* and *goosecoid*, are essential to inhibit the effects of ventralizing BMP and zygotic Wnt proteins, while promoting neural fates (De Robertis, 2009). While creating a zone free of ventralizing factors, the organizer is able to exert its patterning program on the embryo and induce all of the features of the embryonic axis.

The Nodal signaling pathway

The Nodal signaling pathway mediates specification of mesoderm and endoderm, patterning along the dorsal-ventral axis, and patterning along the left-right axis. Ligands of the pathway in *Xenopus* include the maternal Vg1 and Activin proteins, as well as the zygotically expressed *derrière*, and five nodal-related ligands, *xnr1*, *xnr2*, *xnr4*, *xnr5* and *xnr6*. *xnr3* differs from other nodal-related ligands in the frog: it does not demonstrate mesoderm-inducing ability, but instead has neural-inducing ability and is an

important component of the dorsal organizer (Smith et al., 1995; Hansen et al., 1997). The Nodal ligands are first translated as prepro-proteins that must be proteolytically cleaved by the proprotein convertase PACE4 to produce mature bioactive ligands (Eimon and Harland, 2002; Birsoy et al., 2005). Dimerized Nodals signal through a heterodimeric complex of the type I serine-threonine kinase receptor ALK4, ALK5 or ALK7 and the type II receptor ActRII/B; studies using chemical inhibitors of the type I receptors suggest that ALK4 is the primary receptor for Nodal signaling in early zebrafish and *Xenopus* embryos (Reissmann et al., 2001; Ho et al., 2006). Additionally, many Nodal-like ligands require an EGF-CFC co-receptor to confer binding specificity to the type I receptor. In zebrafish, the EGF-CFC co-receptor *oep* is required for the majority of Nodal function (Gritsman et al., 1999). In frog, there are three such receptors, FRL-1, XCR2 and XCR3, and although *xnr1*, *xnr2*, *xnr4*, *xnr5* and *xnr6* require an EGF-CFC co-receptor, Activin and *derrière* do not (Yabe et al., 2003; Dorey and Hill, 2006).

Upon activation, the type II receptor phosphorylates the type I receptor, resulting in the association of Smad2 or Smad3 (Smad2/3) with the type I receptor and subsequent phosphorylation of Smad2/3 by the type I receptor kinase activity (Massague et al., 2005). Smad2/3 then associates with the common mediator (co-Smad), Smad4, and the complex translocates to the nucleus. In frog embryos, this complex then associates with FoxH1 or *Mixer* transcription factors, and mediates target gene expression (Chen et al., 1996; Germain et al., 2000; Kofron, Matt et al., 2004; Kofron, M. et al., 2004). Targets of Nodal signaling include regulators of endodermal and mesodermal cell fate, organizer-specific genes, as well as several positive and negative regulators of the Nodal signaling pathway. These factors work together to facilitate the role of Nodal signaling in specification of endoderm and mesoderm, dorsal-ventral

patterning of the mesoderm, regulation of cell movement during gastrulation and left-right axis patterning.

Regulation of Nodal signaling

In the frog, *xnr5* and *xnr6* are the first Nodals to be expressed and are activated by maternal VegT and β-catenin in the dorsal vegetal quadrant of the embryo. *xnr5* and *xnr6* expression begins in the preMBT embryo by the 64-cell stage, and is restricted to dorsal vegetal cells of the embryo (Yang, Jing et al., 2002; Takahashi et al., 2006). By the MBT, stage 8.5, some *xnr5* and xn6 expression is seen in more lateral regions of the embryo but remains restricted to the vegetal pole; expression begins to decrease by stages 9 to 10, and is lost by the onset of gastrulation at stage 10.5 (Takahashi et al., 2006). Promoter analysis of both genes reveals multiple VegT and β-catenin/TCF binding sites; mutation of the T-box binding sites, maternal depletion of β-catenin or of VegT all lead to loss of *xnr5*/6 expression, demonstrating the requirement for these localized maternal determinants (Takahashi et al., 2000; Rex et al., 2002; Hilton et al., 2003). Interestingly, mutation of the TCF binding sites eliminates the requirement for β-catenin to activate *xnr5* and *xnr6*, allowing for VegT alone to activate expression from these promoters, suggesting that β-catenin serves mainly to relieve the repressive influence of TCF while VegT is the major activator of these ligands (Hilton et al., 2003). Studies of the regulation of *xnr5* and *xnr6* have almost exclusively been in postMBT embryos, although it is known that preMBT expression of *xnr5* and *xnr6* requires β-catenin (Yang, Jing et al., 2002). In this work, I demonstrate that preMBT expression of *xnr5* and *xnr6* requires VegT as well as β-catenin.

Expression of *xnr1*, *xnr2*, *xnr4* and *derrière* are activated at stage 9, hours after activation of *xnr5* and *xnr6* (Jones et al., 1995; Joseph and Melton, 1997; Sun et al.,

1999; Takahashi et al., 2000). All of the Xnrs as well as *derrière* are expressed in vegetal or medial regions of the embryo, with trace or no expression in the animal region. While *xnr1* and *xnr2* are first expressed primarily dorsally, their expression is more medial than the dorsal-vegetal expression pattern of *xnr5* and *xnr6* and by stage 10 their expression domain becomes more ventral (Jones et al., 1995; Agius et al., 2000; Takahashi et al., 2000; Lee, M. A. et al., 2001). *xnr4* is expressed in a much more restricted region in the organizer, and later in the notochord (Joseph and Melton, 1997). *derrière*, a Nodal ligand thought to be involved in posterior development, is first expressed in punctate vegetal cells and throughout the marginal zone of stage 9 embryos, by stage 10.5 is it seen in the marginal zone surrounding the presumptive blastopore by in situ hybridization, with much more intense staining on the dorsal lip (some groups note a dorsal bias in *derrière* expression as early as stage 8.5 (Eimon and Harland, 2002)). By stage 12, *derrière* is expressed exclusively around the blastopore, with the exception of the most dorsal region, which is void of *derrière*; this staining pattern is remarkably similar to that of zygotically expressed VegT (Sun et al., 1999; White et al., 2002). Although *xnr1*, *xnr2*, *xnr4*, *xnr5* and *xnr6* and *derrière* all rely on VegT, only *xnr5* and *xnr6* are reduced in β-catenin depleted embryos (Kofron et al., 1999; Rex et al., 2002; Xanthos et al., 2002). Intriguingly, *derrière*, *xnr1*, *xnr2* and *xnr4*, can all also be induced by Nodal signaling itself, while *xnr5* and *xnr6* cannot be induced by Nodal signaling (Osada et al., 2000; Takahashi et al., 2000; Rex et al., 2002; White et al., 2002).

Nodal signaling is tightly regulated by positive and negative feedback mechanisms. Many Xnrs autoregulate themselves by activating their own expression through FoxH1 binding sites in regulatory regions of these genes, as characterized in detail for *xnr1* (Osada et al., 2000). Nodals also activate expression of extracellular

inhibitors of Nodal function. For example, Lefty is a Nodal ligand antagonist important for the patterning of mesoderm and left-right asymmetry. Lefty is expressed at stage 9 in frog, and overexpression of results in loss of mesoderm (Tanegashima et al., 2000). Lefty competes for binding to the EGF-CFC co-receptor and thus only inhibits the effects of *xnr1*, *xnr2*, *xnr4*, *xnr5* and *xnr6* (Tanegashima et al., 2000; Tanegashima et al., 2004). *Cerberus*-short (Cer-S), a truncated form of the multi-pathway inhibitor and organizer gene *cerberus*, specifically inhibits *xnr1*, *xnr2*, *xnr4*, *xnr5* and *xnr6*, and has been used along with Lefty to examine the effects of Nodal loss of function (Piccolo et al., 1999; Agius et al., 2000).

Nodal signaling and mesoderm development in the frog

Zygotic Nodal expression in the embryo results in the activation of Smad2 by phosphorylation in the vegetal and marginal zones of the embryo that give rise to the endoderm and mesoderm respectively. Interestingly, both the activation of *xnr1*, *xnr2*, and *xnr4* gene expression and the subsequent phosphorylation of Smad2 are localized to the dorsal vegetal region of the embryo shortly after the MBT (Jones et al., 1995; Osada and Wright, 1999; Agius et al., 2000; Takahashi et al., 2000; Lee, M. A. et al., 2001). Indeed, even before the MBT, expression of *xnr5* and *xnr6* ligands begins exclusively in the dorsal-vegetal region of the embryo (Yang, Jing et al., 2002; Takahashi et al., 2006). As development proceeds, P-Smad2 levels are detected in more medial and ventral regions of the embryo, and by the onset of gastrulation the dorsal vegetal region of the embryo no longer contains activated P-Smad2, while P-Smad2 lingers in on the ventral side (Agius et al., 2000; Lee, M. A. et al., 2001; Schohl and Fagotto, 2002). This “wave” of Nodal signaling activity is thought to be the result of the initial early activation of Nodal activity on the dorsal side of the embryo, most likely

due to the localized effect of β-catenin and VegT on *xnr5* and *xnr6* expression. Ventrally, these genes are not expressed and Nodal signaling remains silent. As development continues, intense Nodal signaling activity is gradually attenuated dorsally by autoregulation through Nodal inhibitors, while vegetal regions show increasing Nodal activity due to the contributions of VegT and Vg1 (De Robertis, 2009).

It has been suggested that the dorsal-ventral wave of Nodal activity in the embryo is an important component of not only mesoderm induction, but also mesoderm patterning (Faure et al., 2000; Lee, M. A. et al., 2001). Indeed, the importance of an early gradient of Nodal signaling to patterning the mesoderm has been demonstrated in zebrafish (Gritsman et al., 2000; Chen and Schier, 2001; Hagos and Dougan, 2007; Hagos et al., 2007). In *Xenopus*, several targets of Nodal signaling including *goosecoid*, *chordin* and *Xlim1* are organizer-specific genes, and ectopic expression of *xnr5* can produce a partial secondary axis, suggesting that although Nodals may not be sufficient for organizer formation, they do contribute (Agius et al., 2000; Faure et al., 2000; Takahashi et al., 2000; Takahashi et al., 2006). β-catenin ensures the dorsal expression of these genes in two ways: firstly, β-catenin contributes directly to the activation of organizer genes and secondly, β-catenin contributes to the dorsal activation of *xnr5* and *xnr6* which in turn activate organizer genes through Nodal signaling. Ventrally, lack of β-catenin contributes to the absence of organizer gene expression both directly and through the absence of early Nodal signaling. Additional inhibitory signals from ventral-specific BMP and Wnt signaling contributes to the absence of organizer gene activation ventrally. Although Nodal signaling does occur eventually in the ventral region (at stage 10), it is possible that by that stage of development embryonic cells are no longer competent to respond by producing sufficient levels of organizer genes. In addition to the

anti-organizer effects of ventral cells listed above, this adds a potential temporal competency feature to regulation of mesoderm patterning.

Interestingly, recent work from Blythe and colleagues shows that early dorsal Wnt signaling provides temporal competency to organizer genes, by marking their promoters for activation before the MBT (discussed previously) (Blythe et al., 2010). By stage 10 when intense Nodal signaling occurs ventrally, zygotic wnt signaling has begun ventrally but does not activate organizer gene wnt targets. This may be due in part to the temporal competency conferred by dorsal preMBT Wnt signaling, which marks organizer genes exclusively in dorsal cells for activation after the MBT. Taken together, these observations suggest that early localized Wnt and Nodal signaling both contribute to the dorsal identity of cells in the dorsal-vegetal region of the embryo.

Although signaling through the Nodal pathway is crucial for mesoderm specification and perhaps patterning, the roles of specific Nodal ligands in the early embryo have not been addressed in detail. Recent work from Luxardi and colleagues shows that *xnr5* and *xnr6* together confer a significant portion of mesoderm-inducing activity of Nodals, and that *xnr1* and *xnr2* act later in development to activate movement-effecter genes required for gastrulation movements (Luxardi et al., 2010). Although *xnr5* and *xnr6* are expressed significantly before the MBT, their preMBT contribution to mesoderm specification has not been addressed. Furthermore, the timing of when the vegetal mass of the embryo generates a mesoderm-inducing signal, and when Nodal signaling is first activated is not entirely clear. Although both Ding and colleagues and Jones and Woodland demonstrated that the vegetal mass induces mesoderm in animal pole explants before the MBT (Jones and Woodland, 1986; Ding et al., 1998), other groups have shown that Nodal signaling is not detectable before the MBT (Faure et al., 2000; Lee, M. A. et al., 2001; Saka et al., 2007). In this work, I address the temporal

requirement for Nodal signaling in the embryo and demonstrate that activation of the Nodal signaling pathway by preMBT transcription is necessary for development.

CHAPTER 2:

The maternal transcription factor VegT induces gene expression before the MBT in *Xenopus laevis* embryos[1]

[1] Maternal VegT depletion experiments in this chapter were performed in collaboration with Dr. Jing Yang. Anna-Claire Sienna performed in situ hybridization in this chapter.

SUMMARY

In this chapter I examine the temporal expression pattern of targets of the maternal transcription factor VegT. I find that several genes involved in mesendoderm formation are expressed before the MBT in an RNA Pol II dependent manner, and some are localized to distinct regions in the early embryo. I further characterize their expression through absolute quantitation of transcript number per embryo at various stages before and after the MBT. I also show that VegT is necessary and sufficient for the preMBT expression of many of these genes. In this chapter I describe work that identifies a class of protein coding transcripts activated before the MBT by the maternal T-box factor VegT that are components of an established transcriptional regulatory network required for mesendoderm induction in *Xenopus laevis*.

INTRODUCTION

Following fertilization, early development is primarily under the control of maternal factors until the onset of zygotic gene expression. The timing of the maternal to zygotic transition, which involves degradation of maternal mRNAs and regulated activation of zygotic genes, varies in different organisms (Davidson, 1986; Andeol, 1994; Tadros and Lipshitz, 2009). In *Xenopus*, most zygotic genes are silent until large-scale zygotic gene activation (ZGA) begins at the midblastula transition (MBT), 12 cleavage divisions after fertilization (Newport and Kirschner, 1982a). Although the mechanisms that maintain transcriptional silence before the MBT are not well characterized, early *Xenopus* embryos contain the components needed for RNA polymerase-II-dependent transcription (Veenstra, 2002),

Despite the global transcriptional quiescence of the zygotic genome during cleavage stages, transcription of a small number of protein coding genes before large

scale ZGA has been uncovered in many organisms including fly, frog, zebrafish, and mouse (reviewed in (Andeol, 1994; Tadros and Lipshitz, 2009). In *Xenopus*, the Nodal-related genes *xnr5* and *xnr6* are transcribed up to 6 cell divisions before the MBT (Yang et al., 2002; Takahashi et al., 2006; Rosa et al., 2009; Blythe et al., 2010), and additional high molecular weight polyadenylated RNAs are also transcribed before the MBT (Nakakura et al., 1987; Yang et al., 2002). Although the regulation of *xnr5* and *xnr6* before the MBT is dependent on β-catenin, little is known about contribution from other transcription factors or if other genes are expressed before the MBT in *Xenopus* (Yang, Jing et al., 2002).

The presence of preMBT transcription in diverse organisms suggests that this early transcription may be specifically regulated and serve a developmental function. In *Drosophila*, the promoters for most preMBT genes contain a heptad repeat that regulates preMBT transcription (ten Bosch et al., 2006; De Renzis et al., 2007). Increasing the number of heptad repeats accelerates expression and removing the heptad repeats delays expression (ten Bosch et al., 2006). This cis-acting element binds the Bicoid Stability Factor (BSF), the zinc finger protein Zelda, and Grainyhead (De Renzis et al., 2007; Liang et al., 2008; Harrison et al., 2010). Furthermore, maternal loss of Zelda globally interferes with preMBT transcription and results in defects in cellularization soon after the MBT (Liang et al., 2008). These observations indicate that a site-specific, activating factor is required (in *Drosophila*) for preMBT transcription in an otherwise repressive context. Although a similar regulator has not been identified in *Xenopus* or other vertebrates, we report here that VegT, a maternal T-box transcription factor required for the induction of mesoderm and endoderm, regulates the preMBT expression of multiple genes in *Xenopus*.

Expression of *xnr5* and *xnr6* before the MBT requires maternal Wnt signaling (Yang, Jing et al., 2002). Transcription of *xnr5* and *xnr6* after the MBT also requires the maternal T-box transcription factor VegT (Hilton et al., 2003); we therefore investigated the contribution of VegT to preMBT transcription. We find that multiple VegT target genes, in addition to *xnr5* and *xnr6,* are transcribed well before the MBT in *Xenopus*. All of these genes were previously identified as components of a gene regulatory network involved in the induction of endoderm and mesoderm (Zorn and Wells, 2007). Maternal loss-of-function and gain-of-function data show that VegT is necessary and sufficient for the preMBT expression of these genes.

MATERIALS & METHODS

Xenopus Embryo Manipulations

Embryos were obtained by in vitro fertilization, cultured, and injected as described (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryos were maintained at 23ºC and the intervals between the first 8 cell divisions were closely monitored for each clutch of eggs. The timing of the 10^{th} through the 12^{th} cleavage divisions was predicted based on the length of the earlier cell cycles (approximately 22 minutes at 23ºC) and consistency in staging between different clutches was ensured by photographing embryos at each stage. Animal cap explants were excised and cultured as described (Sive et al., 2007). Dorsal-ventral dissections were performed by first injecting ventral blastomeres (determined by pigmentation) at the 4-cell stage with fluorescent dextran and then manually dissecting 256-cell stage embryos with a hair knife under epifluoresence.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed on staged embryos as described (Blythe et al., 2009). Briefly, proteins and nucleotides were crosslinked by a 1 hour incubation in 1% formaldehyde in PBS (phosphate buffered saline). Embryos were then lysed and chromatin was sheared by sonication. Lysates were incubated with 5uL anti-myc antibody (Blythe et al., 2009), and pulled down with protein G agarose. DNA products were purified, and subjected to nested PCR analysis using the primers listed in Table 2.0.

mRNA Injection

VegT was amplified by PCR and cloned into pCS2-MT. MT-VegT was linearized with Not I and synthetic capped mRNA was produced with the mMessage mMachine kit (Ambion, Austin, TX, USA) using SP6 polymerase.

Morpholinos and VegT Maternal Depletion

Antisense morpholino oligonucleotide (MO) targeting β-*catenin* (Heasman et al., 2000) was purchased from GeneTools LLC (Philomath, OR, USA). Maternal depletion of VegT was carried out as described (Zhang et al., 1998). Briefly, full-grown oocytes were manually defolliculated and cultured in oocyte culture medium. Oocytes were vegetally injected with 5 ng of oligo (C*A*G*CAGCATGTACTT*G*G*C). Oocytes were cultured for 24 hours at 18°C, stimulated with 2 μM progesterone overnight, stained with dyes, and transferred into foster mothers. Two hours after the egg transfer, the host was placed in 1XMMR. After 4 hours, all eggs were collected and fertilized using a sperm suspension. Colored experimental embryos were sorted out and cultured in 0.2XMMR.

RT-PCR and qRT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on staged embryos as described (Blythe et al., 2010), except that non-radioactive nucleotides were used and PCR products were separated via gel electrophoresis on a 2.5% agarose gel and visualized with ethidium bromide. cDNA was synthesized using random primers (Yang et al., 2002). Primers are described in Table 2.1.

Quantitative RT-PCR (qRT-PCR) was performed as described (Blythe et al., 2009). For each experiment, individual samples were analyzed in triplicate. Unless otherwise noted, data were analyzed by first normalizing to ODC loading control, and then calculating fold change compared to the indicated control condition using the ΔΔC(t) method (reviewed in (Taneyhill and Adams, 2008)). Embryonic cDNA was synthesized as described above, using random primers. To measure the number of transcripts per embryo, a standard curve of cDNA for each marker was analyzed in parallel with cDNA derived from embryos at different stages. For each primer set, a serial dilution of the plasmids encoding each target gene (10^4, 10^3, 10^2, and 10^1 copies) was subjected to qPCR in parallel with qRT-PCR for each sample. For the standard curve, C(t) was plotted as a function of copy number, and linear regression was used to determine copy number for a given sample based on the results of the standard curve. The number of embryos per sample for the qRT-PCR reaction was determined based on the starting number of embryos, and the final copy number result was adjusted according to the total number of embryos each template sample represents. The plasmids used in the standard curve were as follows: pBSK-*Bix4* (IMAGE clone from Open BioSystems, GenBank: BG022838.1); pCS2-*derrière* (Sun et al., 1999); pCS2-*xnr5* (described below); pBSK-*xnr6* (Takahashi et al., 2000); pCS2-chordin (Sasai et al., 1994) pBSK-edd (Sasai et al., 1996). The *xnr5* open reading frame was PCR-amplified

from pNRRX-*xnr5* (Takahashi et al., 2000) and cloned into pCS2 to make pCS2-*xnr5*. qPCR primers are described in Table 2.2.

In situ hybridization

Whole-mount *in situ* hybridization (WISH) with digoxygenin labeled probes (Roche, Indianapolis, IN, USA) was carried out as described (Deardorff et al., 1998). For anti-sense *xnr3* probe, pGEM5Zf(-)-*xnr3* was linearized with EcoRI and transcribed with T7 polymerase in the presence of digoxygenin-labeled UTP.

RESULTS

VegT can associate with a target promoter before the MBT

During *Xenopus* development, the Nodal-related genes *xnr5* and *xnr6* are expressed before the midblastula transition (MBT) (Yang, Jing et al., 2002; Takahashi et al., 2006; Rosa et al., 2009; Blythe et al., 2010; Luxardi et al., 2010). Although the β-catenin-TCF transcription complex, a key modulator of Wnt-dependent transcription, is necessary for this preMBT gene expression (Yang, Jing et al., 2002; Blythe et al., 2010), other Wnt target genes, such as *siamois*, *twin*, *myf5*, and *engrailed*, are not expressed before MBT, suggesting that activity of the β-catenin-TCF complex is not sufficient to drive preMBT transcription. The maternal transcription factor VegT is also required for expression of *xnr5* and *xnr6*; although this has so far only been determined for *xnr5* and *xnr6* expression after the MBT (Takahashi et al., 2000; Rex et al., 2002; Xanthos et al., 2002), we considered the possibility that VegT could be the maternal factor that determines preMBT expression of these two genes. We first examined the ability of VegT to associate with target promoters before the MBT. Indeed, when chromatin immunoprecipitation (ChIP) was performed on 1000 cell stage embryos injected with

50pg of MT-VegT, MT-VegT was found in to preferentially associate with the *xnr6* promoter and did not associate with a control promoter (xMLC) (Fig. 2.0).

VegT target genes are transcribed before the midblastula transition

As VegT directly regulates other mesendodermal genes and can be detected in the nucleus by the 16-cell stage (Stennard et al., 1999), we examined whether other VegT targets are transcribed prior to the MBT in a manner similar to *xnr5* and *xnr6*. We isolated mRNA from multiple pre and post MBT stages and assessed gene expression using RT-PCR. The VegT targets *derrière*, *bix4*, *mixer,* and *sox17*α are newly transcribed before the MBT, with the first new transcripts appearing between the 256 and 1000-cell stages (Fig. 2.1 A). Similar data was obtained for vent1 (data not shown). In contrast, the direct VegT target genes *chordin* and *endodermin* are readily detectable after the 4000-cell stage (MBT).

We also measured the number of mRNA transcripts per embryo for several of these preMBT genes using quantitative reverse transcription-coupled PCR (qRT-PCR) to compare mRNA isolated from staged embryos to a standard curve for each gene (Fig. 2.1 B). We found a baseline of approximately 10^2 transcripts per embryo at the 4-cell stage for most of the mRNAs, although *xnr6* was considerably lower. The number of transcripts for the preMBT genes *xnr5, xnr6, derrière*, and *bix4* increased from 3 to 70-fold from the 4-cell to the 256-cell stage and another 5 to 20-fold from the 256-cell to 1000-cell stage (Fig. 2.1 B). In contrast, *chordin* and *endodermin* remained at baseline throughout preMBT stages, with *chordin* rising sharply at the 4000-cell stage (MBT) and *endodermin* increasing after the MBT. The number of transcripts/embryo for *mixer* and *sox17*α also increased 2 to 5-fold between the 256-cell and 1000-cell stage, although *sox17*α transcript levels were initially relatively high, even at the 4-cell stage, suggesting

a low level maternal contribution to *sox17α* expression (Howard et al., 2007)(data not shown). To demonstrate that the appearance of these transcripts depends on new transcription, we measured mRNA levels from preMBT embryos with or without α-amanitin, an RNA polymerase II (pol II) inhibitor (Fig. 2.2 A); we found that all preMBT transcripts were greatly reduced in the presence of α-amanitin, although some *sox17α* remained, presumably representing maternal mRNA. In summary, these data show a rapid, pol II-dependent increase in the number of mRNA transcripts for preMBT genes detectable as early as the 256-cell stage and clearly distinct from typical zygotic genes that are not transcribed until the MBT.

Before the MBT, *xnr5* and *xnr6* are expressed predominantly in dorsal-vegetal blastomeres (Yang, Jing et al., 2002; Takahashi et al., 2006). To test whether dorsal expression is a common feature of other preMBT genes, we dissected 1000-cell embryos into dorsal and ventral halves, isolated RNA, and performed qRT-PCR. Similar to *xnr5* and *xnr6* expression, *bix4* and *derrière* are preferentially expressed in the dorsal half of the preMBT embryo, whereas new *sox17α* and *mixer* transcripts are equally distributed (Fig. 2.2 B). *vent1* was distributed in a pattern similar to *sox17α* and *mixer* (data not shown). Thus, preMBT transcription is not restricted to dorsal-vegetal cells, consistent with the widespread distribution of maternal VegT protein in the vegetal hemisphere (Horb and Thomsen, 1997).

To further examine the spatial distribution of preMBT transcription, we dissected 1000-cell embryos into left and right halves, isolated RNA, and performed RT-PCR. All of the preMBT transcripts analyzed showed similar levels of distribution across the left-right axis Fig 2.2 C)

The divergent TGFβ member *xnr3* is expressed before the MBT

Because the preMBT genes *xnr5* and *xnr6* are members of a larger group of related signaling molecules, we examined the expression pattern for all known Xnr genes in the embryo, *xnr1*, *xnr2*, *xnr3*, *xnr4* as well as the preMBT genes *xnr5* and *xnr6* (Fig. 2.3). Remarkably, although transcripts of *xnrs 1, 2* and *4* only begin accumulating at the MBT, we found that transcripts of the divergent Xnr gene *xnr3* began to accumulate well before the MBT, in a pattern very similar to *xnr5* and *xnr6*. Since many previous reports show *xnr3* expression begins after the MBT, and the early expression pattern we observed was quite robust and reproducible, we decided to verify the early expression pattern. Inhibition of transcription with the RNA Pol II inhibitor α-amanitin confirmed that the preMBT *xnr3* signal we observed was indeed the result of transcription before the MBT (Fig. 2.4 A). To confirm that the PCR product observed was indeed *xnr3*, the product was digested with the restriction enzyme RsaI which should uniquely cleave the 238bp product into 146bp and 92bp products. Digestion of the *xnr3* PCR product followed by gel electrophoresis resulted in a larger band and a smaller band corresponding to 146bp and 92bp respectively (Fig. 2.4 B), suggesting that the PCR product we observed was likely that of *xnr3*.

To further confirm the finding of preMBT *xnr3* expression, we performed RT-PCR on a timecourse of staged embryos using at least five different *xnr3* primer sets, all of which correspond to different areas along the length of the *xnr3* sequence and all of which have been previously published (Table 2.3) (Fig. 2.5A). We performed the PCR for each primer set on a cDNA timecourse in concert with a standard curve of *xnr3* plasmid, and thus were able to detect any variations in primer set sensitivity by analyzing the signal for the lowest copy number of *xnr3* plasmid each primer set was able to detect. We confirmed the finding that *xnr3* is detectable before the MBT for all of the

published primer sets. Not surprisingly, we noted that primer sets showing the earliest detection of *xnr3* transcripts corresponded to the primer sets that proved to be the most sensitive in detecting low-levels of the plasmid standard curve (Fig. 2.5 B).

To further characterize the expression of *xnr3* before the MBT, we performed quantitative RT-PCR analysis along side a plasmid standard curve for absolute quantitation, as described in Figure 2.1 B and the Methods section. We found that *xnr3* transcripts increased 55-fold from the 4 cell to the 256 cell stage, and a further 10-fold from the 256 cell stage to the 1000 cell stage (Fig. 2.6 A). Calculated numbers of transcripts per embryo for *xnr3* is listed, along with *endodermin* (postMBT gene) and *xnr5* and *xnr6* (preMBT genes) for comparison (Fig. 2.6 B). *xnr3* transcripts accumulate to a similar extent as *xnr5* and *xnr6* before the MBT; in contrast, *endodermin* transcripts do not accumulate before the MBT. We were also able to determine that inhibition of transcription by injection of α-amanitin decreased *xnr3* expression at the 1000 cell stage (preMBT) by >99.8% (Fig. 2.6 C). *xnr3* requires maternal β-catenin for expression; accordingly, when β-catenin was depleted from embryos using antisense morpholino oligonucleotides, expression of *xnr3* at the 1000 cell stage was reduced by >98% (Fig. 2.7 A). *xnr3* expression is also restricted to the dorsal marginal region of the embryo after the MBT. By dissecting embryos into dorsal and ventral halves and analyzing by qRT-PCR (as in Figure 2.2 B), we found that preMBT expression of *xnr3* is restricted to the dorsal half of the embryo (Fig. 2.7 B). Additionally, we confirmed preMBT dorsal expression of *xnr3* by in situ hybridization (Fig 2.7 C). Taken together, these data suggest that *xnr3* is a preMBT gene in *Xenopus* embryos. The implications for this finding will be discussed in more detail in the discussion section of this chapter and in Chapter 4.

VegT is required for expression of many preMBT genes

To determine whether maternal VegT is necessary for preMBT gene expression, we depleted *VegT* mRNA from oocytes using antisense phosphorothioate oligos and generated *VegT*-deficient embryos using the host-transfer method (Heasman et al., 2000). Control and *VegT*-depleted embryos were harvested at the 1000-cell stage (2 cell cycles before the MBT) for qRT-PCR. Expression of *bix4*, *derrière*, *xnr5*, and *xnr6* was reduced by 64% - 85% in *VegT*–depleted embryos (Fig. 2.8 A), demonstrating that maternal *VegT* is required for the preMBT expression of these genes. *sox17*α also requires VegT, but was reduced by only 46% in *VegT*-depleted embryos, reflecting the maternal contribution seen in Figure 1. To test whether *VegT* is sufficient to induce preMBT gene expression, we expressed *VegT* in animal pole cells, which normally do not express this vegetally localized RNA. Ectodermal explants were excised at the 1000-cell stage and RNA was isolated for qRT-PCR. As shown in Figure 2B, VegT induces preMBT transcription of the established VegT target genes *bix4*, *derrière*, *mixer*, *sox17a,* *xnr5,* and *xnr6* in animal cap explants in a dose-dependent manner. Interestingly, VegT did not induce precocious expression of *chordin* or *endodermin*, VegT targets that are normally not expressed until after the MBT (Fig. 2.8 B).

Although expression of *xnr5* and *xnr6* requires maternal β-catenin as well as VegT, the other preMBT VegT target genes identified here are not dependent on β-catenin. Thus, when β-catenin was depleted with an antisense morpholino oligonucleotide (Heasman et al., 2000), preMBT expression of these VegT target genes was not affected, whereas expression of *xnr5* and *xnr6* was reduced by 85% and 93%, respectively (Fig. 2.8 C). Together, these data show that VegT is required for preMBT transcription of multiple target genes.

DISCUSSION

The finding that a distinct class of mesodermal and endodermal regulators is expressed before the MBT raises interesting questions including how these genes escape transcriptional silencing, if their preMBT expression is required for development, and what their early developmental role, if any, might be. These genes are all regulated either directly (*xnr5, xnr6, sox17β, vent1,* and *bix4*) or indirectly (*mixer*) by VegT (Taverner et al., 2005). However, not all VegT targets are expressed before the MBT. Several targets, including *endodermin* and *chordin* are not expressed until after the MBT (Fig. 2.1). This may reflect a requirement for a secondary activating factor that binds preMBT but not postMBT VegT targets to mediate pre- vs. postMBT gene expression. Conversely, this observation could reflect the presence of a transcriptional inhibitor associated with post- and not preMBT VegT targets; release of this repression by some mechanism at the MBT would then allow VegT to activate *chordin* and *endodermin*. In either scenario, the requirement for VegT represents a common factor in the preMBT expression of all of the genes described in this chapter (with the possible exception of *xnr3*, discussed below).

xnr5, *xnr6*, *sox17α*, *bix4*, *derrière* and *vent1* all contain T-box binding sequences in their promoters, presumably the site through which VegT mediates their activation (Casey et al., 1999; White et al., 2002; Hilton et al., 2003; Taverner et al., 2005; Howard et al., 2007; Blythe et al., 2009). Although this site may not be analogous to the TAGteam site of preMBT *Drosophila* genes, it nevertheless represents a specific sequence domain conserved among preMBT genes with the possible exception of *mixer*, which is an indirect target of VegT (Taverner et al., 2005). Another possible exception to the observation that preMBT genes contain T-box binding sites is *xnr3*. In contrast to *xnrs 1, 2, 4, 5* and *6*, all of which are upregulated synergistically by VegT and

β-catenin in animal cap assays, *xnr3* is not upregulated by VegT (Rex et al., 2002). Interestingly, the presence of exogenous VegT in animal cap assays abrogates the ability of β-catenin to induce *xnr3*, suggesting that VegT can act as a negative regulator of this gene. A search of *X. laevis* and *X. tropicalis xnr3* promoters revealed a core T-box binding sequence upstream of the ATG of each gene (see Chapter 4 for details).

The expression of *xnr3* before the MBT is surprising since previous reports examining preMBT transcription have not detected this expression, however this may be due to highly variable primer efficiency as demonstrated in Figure 2.5. The possibility that *xnr3* is regulated by VegT is also surprising. Indeed, preliminary experiments done during this thesis suggest that VegT can associate with the *xnr3* promoter in preMBT embryos, data consistent with VegT mediating transcriptional regulation of *xnr3* (Fig. 2.0). However some VegT depletion studies have demonstrated that *xnr3* expression requires maternal VegT, a finding that suggests a more complicated relationship between VegT and *xnr3* (Kofron et al., 1999; Xanthos et al., 2002). For example, it is formally possible that *xnr3* requires a downstream VegT target for activation in the marginal zone, while VegT itself limits the expression of *xnr3* in the vegetal domain of the embryo, by binding to and inhibiting the *xnr3* promoter. The expression pattern of *xnr3* and maternal VegT support this possibility, since they occupy non-overlapping areas of the embryo (*xnr3* expression is exclusively marginal while VegT expression is vegetally restricted). Additionally, depletion studies show that *xnr3* expression is, in part, dependent on FoxH1; therefore it is possible that vegetally localized VegT is required to activate Nodal signals in the overlying mesoderm, which in turn activate *xnr3* expression via the Nodal transcriptional mediator FoxH1.

With the exception of the organizer gene *xnr3*, the preMBT genes identified in this study are all components of the mesendoderm specification mechanism in embryos,

and are all expressed in the vegetal and/or marginal zone at late blastula stages. *bix4* is a member of the Bix family of homeobox transcription factors that plays an important role in endoderm induction. *bix4* is a direct target of VegT and is capable of rescuing endoderm-specification in VegT depleted embryos, although not mesoderm formation (Casey et al., 1999). *mixer* is also a homeodomain transcription factor and is essential for endoderm formation. Expression of *mixer* mRNA is restricted to the endoderm at the boundary of prospective endoderm and mesoderm, and loss of function studies suggest that *mixer* plays a role in defining the border between endoderm and mesoderm (Engleka et al., 2001; Kofron, M. et al., 2004). *mixer* induces endoderm in animal caps and plays an important role in negatively regulating mesodermal gene expression in the embryo (Henry and Melton, 1998; Sinner et al., 2006). *vent1* is also a homeobox domain transcription factor, and is expressed primarily on the ventral side of the embryo where it mediates ventral mesoderm identity through the negative regulation of dorsally localized *goosecoid* (Gawantka et al., 1995; Sander et al., 2007). Although *vent1* is primarily a target of ventral BMP signaling, there are as many as eight T-box binding sites in its promoter, and it is strongly upregulated by VegT in animal cap assays (Gawantka et al., 1995; Taverner et al., 2005). *sox17*α is an HMG box domain transcription factor important for endoderm specification. It is a direct target of VegT, and is involved in multiple regulatory feedback loops with other endodermal genes as well as Nodals and itself (Sinner et al., 2004; Sinner et al., 2006; Howard et al., 2007).

derrière is a Nodal-like ligand and a component of the TGFβ signaling pathway, and can induce a wide range of mesoderm and endoderm markers. Dorsal misexpression of *derrière* results in a loss of head structures whereas ventral overexpression causes partial posterior secondary axis formation (Sun et al., 1999). Although *derrière* is expressed throughout the marginal zone in early embryos, and

excluded from the dorsal most region of the blastopore by stage 12, some groups have reported a dorsal bias in *derrière* expression in the vegetal pole of midblastula embryos (Sun et al., 1999; Eimon and Harland, 2002; White et al., 2002). This is consistent with the dorsal preference for *derrière* expression we observe in preMBT embryos (Fig. 2.2).

xnr5 and *xnr6* are also Nodal-like ligands belonging to the Xnr family of genes. Xnr genes are exclusively zygotically expressed in the embryo, and although this group as a whole is known to be important for mesoderm induction, the contributions of individual Xnr genes have not been well characterized. *xnr5* and *xnr6* have previously been reported as expressed in the preMBT embryo in the dorsal vegetal region (Takahashi et al., 2000; Yang, Jing et al., 2002; Takahashi et al., 2006). Both genes can induce mesodermal and endodermal markers, as well as secondary axes when overexpressed in whole embryos, suggesting they play a role in mesendoderm specification and axis formation (Takahashi et al., 2000; Onuma et al., 2002). Cleavage mutants for *xnr5* or *xnr6*, which are incapable of proteolytic cleavage to their active forms, cause severe defects in the embryo including loss of mesoderm formation, shortened body axis, loss of dorsal structures and gastrulation defects (Onuma et al., 2002). Although it is not entirely clear if these effects are due to specific loss of *xnr5* and *xnr6* function since these cleavage mutants may precociously interact with and inhibit other Xnrs, it nonetheless highlights the important role that Xnrs play in the early embryo.

A recent study from the Kodjabachian group sought to determine the function of individual Xnrs to early development by targeting each for inhibition with antisense morpholino oligonucleotides (Luxardi et al., 2010). The results of this study established that *xnr5* and *xnr6* are primarily responsible for mesoderm induction and the activation of *xnr1* and *xnr2* expression, whereas *xnr1* and *xnr2* expression are primarily responsible

for the activation of movement-effecter genes which mediate cell movements during gastrulation. The results of this study suggest that *xnr5* and *xnr6* may mediate mesoderm induction in the preMBT embryo, although the temporal requirement for *xnr5* and *xnr6* has not yet been determined.

The regulated activation of preMBT genes suggests that they may play an important role in development of the embryo. In the next chapter, I describe experiments which determine that preMBT expression of *xnr5* and *xnr6* are required for activation of Nodal signaling at midblastula stages. By inhibiting Nodal signaling at various times during development, I also determine that Nodal signaling is required during preMBT stages for normal development to occur.

FIGURES AND FIGURE LEGENDS

Table 2.0: ChIP Primer Sequences

Primer name	Sequence	Notes
Xnr6 outer Fwd	5'- TCT GAG GTG TGA GGT ATA TGA AAG G -3'	Blythe et al., 2009
Xnr6 outer Rev	5'- TGG GGC TCT TGA AAA CTG AAA TG -3'	Blythe et al., 2009
Xnr6 inner Fwd	5'- GGT AGA TGA AAG GCT GAC AGG TGT G -3'	Blythe et al., 2009
Xnr6 inner Rev	5'- GGC TGT TGA AAA CTG AAA TGA AGC -3'	Blythe et al., 2009
Xnr3 outer Fwd	5'- ATA GCT TTA ATG TGC CAC AAT CTA -3'	Blythe et al., 2009
Xnr3 outer Rev	5'- GTA CAG TCT TGG GAG TTC CCT G -3'	Blythe et al., 2009
Xnr3 inner Fwd	5'- CAT AAA GGC AAA TGG TTT CTG C -3'	Blythe et al., 2009
Xnr3 inner Rev	5'- TTA TAC TGG GAT GGA CAG AGG C -3'	Blythe et al., 2009
xMLC outer Fwd	5'- TGG GAT ATT TTA CTG AAC ACA ATG -3'	Blythe et al., 2009
xMLC outer Rev	5'- CGT CCT GTG CCA CCT AAT G -3'	Blythe et al., 2009
xMLC inner Fwd	5'- GAA TGT TAG CCC TTG TGC TCT T -3'	Park et al., 2005
xMLC inner Rev	5'- GGA AAG TTC TCT TGA TCA TTT TA -3'	Park et al., 2005

Table 2.1: RT-PCR Primers

Primer	Sequence	Source
endodermin Fwd	5'- AGC AGA AAA TGG CAA ACA CAC -3'	Klein Lab
endodermin Rev	5'- GGT CTT TTA ATG GCA ACA GGT -3'	
chordin Fwd	5'- ACA GCA TAG GCA GCT GTG -3'	Sasai et al., 1994
chordin Rev	5'- GTG TGC TTG GAC AAG AGG -3'	
mixer Fwd	5'- CAC CAG CCC AGC ACT TAA CC -3'	Klein Lab
mixer Rev	5'- CAA TGT CAC ATC AAC TGA AG -3'	
bix4 Fwd	5'- AGA TGC TAC AGG CTG GAG CAA -3'	Casey et al., 1999
bix4 Rev	5'- GTG TGT AAG GGG TGA GTC ATA -3'	
derriere Fwd	5'- TGG CAG AGT TGT GGC TAT CA -3'	Sun et al., 1999
derriere Rev	5'- CTA TGG CTG CTA TGG TTC CTT -3'	
xnr5 Fwd	5'- TCA CAA TCC TTT CAC TAG GGC -3'	Yang et al., 2002
xnr5 Rev	5'- GGA ACC TCT GAA AGG AAG GC -3'	
xnr6 Fwd	5'- TCC AGT ATG ATC CAT CTG TTG C -3'	Yang et al., 2002
xnr6 Rev	5'- TTC TCG TTC CTC TTG TGC CTT -3'	
sox17α Fwd	5'- CAG GTG AAG AGG ATG AAG AG -3'	Klein Lab
sox17α Rev	5'- GCT GGA GAT GTG AAG AAC AC-3'	
odc Fwd	5'- AAT GGA TTT CAG AGA CCA -3'	Klein Lab
odc Rev	5'- CCA AGG CTA AAG TTG CAG -3'	
vent1 Fwd	5'- GCA TCT CCT TGG CAT ATT TGG -3'	Gawantka et al., 1995
vent1 Rev	5'- TTC CCT TCA GCA TGG TTC AAC	
xnr1 Fwd	5'- TGG CCA GAT AGA GTA GAG -3'	Kofron et al., 1999
xnr1 Rev	5'- TCC AAC GGT TCT CAC TTT -3'	
xnr2 Fwd	5'- ATC TGA TGC CGT TCT AAG CC -3'	Takehashi et al., 2000
xnr2 Rev	5'- GAC CTT CTT CAA CCT CAG CC -3'	
xnr3 Fwd	5'- TGA ATC CAC TTG TGC AGT TCC -3'	Ding et al., 1998
xnr3 Rev	5'- GAC AGT CTG TGT TAC ATG TCC -3'	
xnr4 Fwd	5'- TTA CAA GAT GCT GCA CAC TCC -3'	Takehashi et al., 2000
xnr4 Rev	5'- AAC TCT GCA TGT ATG CGT GG -3'	

Table 2.2: qRT-PCR Primers

Primer	Forward	Reverse
bix4	TTCTCCTCCAGTGACCACCT	TTCCCCAGCCTTCATGTCT
derriere	CAGGAAGCTCCTGGTGACTC	CAAATGATCGATTGCCTCCT
xnr5	GGCCTGTGGGAGATGATAAA	TAATCAGGTCTCCCCAACCA
xnr6	CTCTCATCATGGGGAATGCT	CTGTCCAGGCCAAAGGATAA
chordin	CAGCTGCAAAAACATCAAACA	CAAGTCTTGCAGCAATGTCC
endodermin	CTCGCTCTGGACAAAACTCC	TCATTCAGAGCGATTTGCTG
odc	GATCATGCACATGTCAAGCC	TCTACGATACGATCCAGCCC
mixer	CTTCAACAACTTCCCTCCCA	CATCCGACTGTGGTTGTTTG
sox17α	TCCTCTGGATTTGGCAGAAG	GTGAGGGTAGCTGCCATTGT
xnr3	GGGGAACTTCCTTCCTTGAA	CTGTGGAACTGCACAAGTGG
vent1	TCAGCCCCGGGCACTTCCAT	TGAGCCCCAAAGAGTGGGGGA

Table 2.3: Xnr3 RT-PCR Primers

Primer	Sequence
a Fwd: Ding et al., 1998	TGAATCCACTTGTGCAGTTCC
a Rev: Ding et al., 1998	GACAGTCTGTGTTACATGTCC
b Fwd: Agius et al, 2000	CGAGTGCAAGAAGGTGGACA
b Rev: Agius et al., 2000	ATCTTCATGGGGACACAG
c Fwd: Kofron 2001	CTTCTGCACTAGATTCTG
c Rev: Kofron 2001	CAGCTTCTGGCCAAGAACT
d Fwd: Kofron et al., 2004	TAATCTGTTGRGCCGATCCA
d Rev: Kofron et al., 2004	ATCAATGTTGCCCTTTTTCA
e Fwd: Nelson and Gumbiner 1999	ATGGCATTTCTGAACCTG
e Rev: Nelson and Gumbiner 1999	TCTACTGTCACACTGTGA

Figure 2.0: VegT can bind to a Nodal target promoter before the MBT.

Chromatin immunoprecipitation (ChIP) was performed on 1000 cell stage embryos (preMBT, stage 7). Embryos were injected with 50pg myc-tagged VegT (MT-VegT) uilaterally at the 1-cell stage, cultured until the 1000 cell stage, and fixed as described. Prepared embryo extracts from injected and not injected embryos were subjected to immunoprecipitation with anti-myc antibody. Nested PCR was performed on precipitated DNA or input samples for genomic *xnr6*, *xnr3* or xMLC as described. Input samples represent 0.1% or 1% of total input for injected or not-injected embryos lysates, as indicated.

Figure 2.0

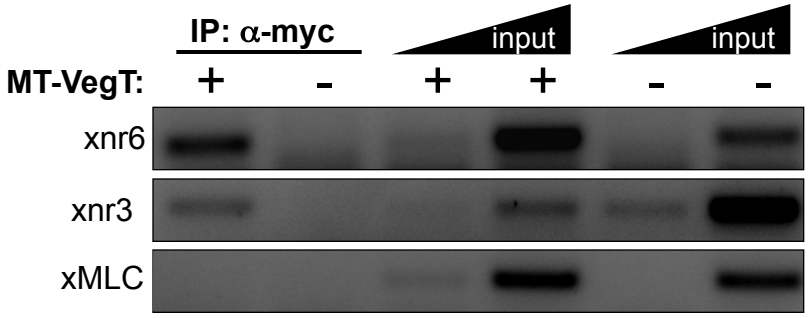


Figure 2.1: Mesendodermal genes transcribed before the MBT.

(A) RT-PCR was performed on embryos collected at the indicated stages. 35 cycles were used for all samples except for *sox17*α (30 cycles) and *odc* (23 cycles).

(B) Quantitative RT-PCR was performed on staged embryos and the number of transcripts/embryo was determined as described in Materials and Methods. Log_{10}[transcripts/embryo] was plotted for each gene. MBT = 4000c; -rt = no reverse transcriptase.

Figure 2.1

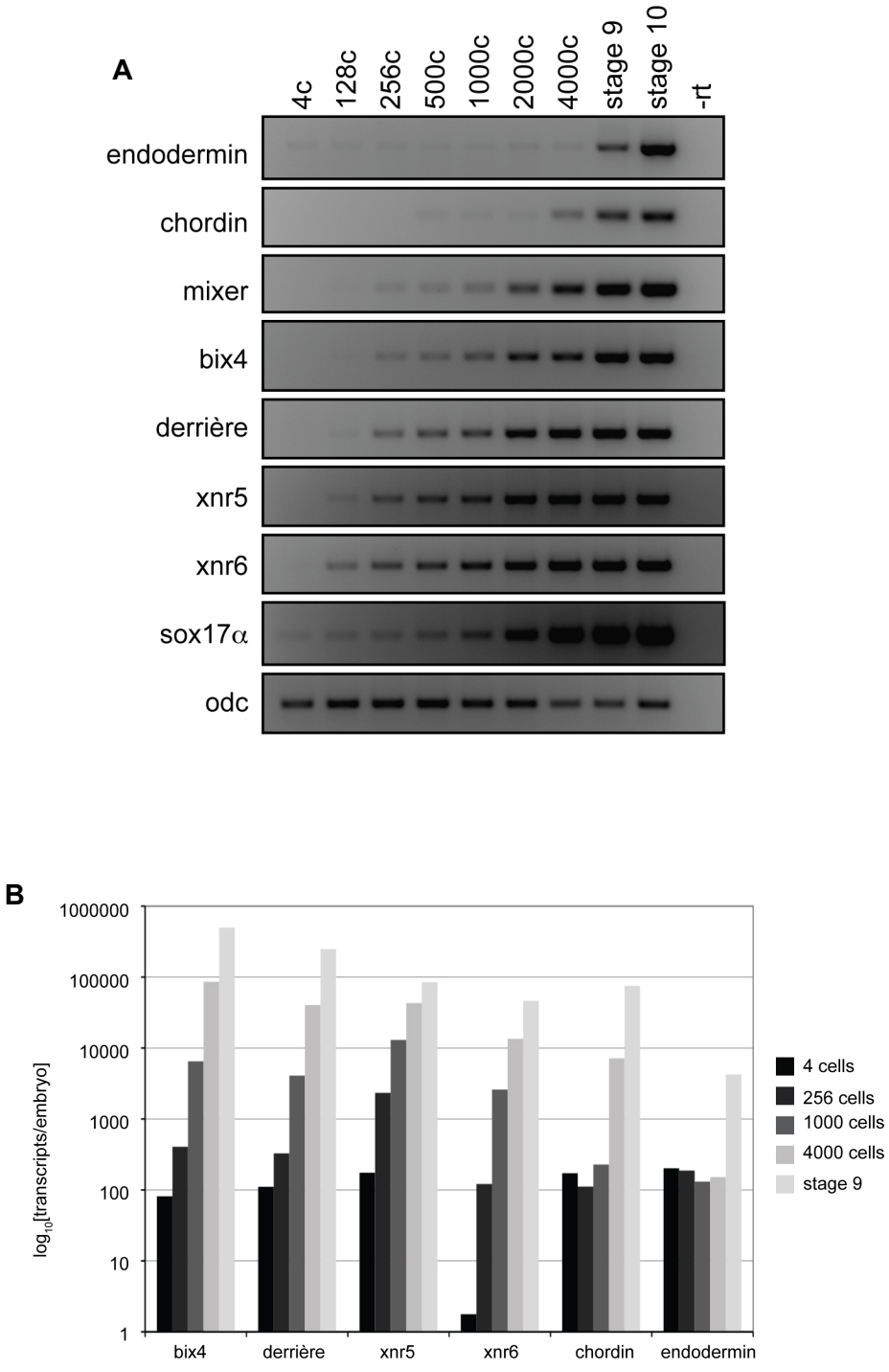

A
4c
128c
256c
500c
1000c
2000c
4000c
stage 9
stage 10
-rt
endodermin
chordin
mixer
bix4
derrière
xnr5
xnr6
sox17α
odc
B
1000000
100000
10000
1000
100
10
1
log10[transcripts/embryo]
bix4
derrière
xnr5
xnr6
chordin
endodermin
4 cells
256 cells
1000 cells
4000 cells
stage 9

Figure 2.2: preMBT mesendodermal genes are dependant on RNA Pol II and many are dorsally localized.

(A) α-amanitin (100pg/embryo) was injected into both cells at the 2-cell stage. Injected and non-injected controls were harvested at the 1000-cell stage (preMBT) for qRT-PCR analysis. The results were normalized to expression in the absence of α-amanitin (ni) for each gene. ni, not injected. Error bars represent standard error of the mean for 2 or more experiments.

(B) Two ventral cells at the 4-cell stage were injected with rhodamine dextran as a lineage tracer, dissected into dorsal and ventral halves at the 256-cell stage, then harvested at the 1000-cell stage for qRT-PCR. Error bars represent standard error of the mean for 2 or more experiments.

(C) Embryos injected as in (B) were dissected along the dorsal and ventral axis at the 256-cell stage, then left or right halves were harvested at the 1000-cell stage for qRT-PCR

Figure 2.2

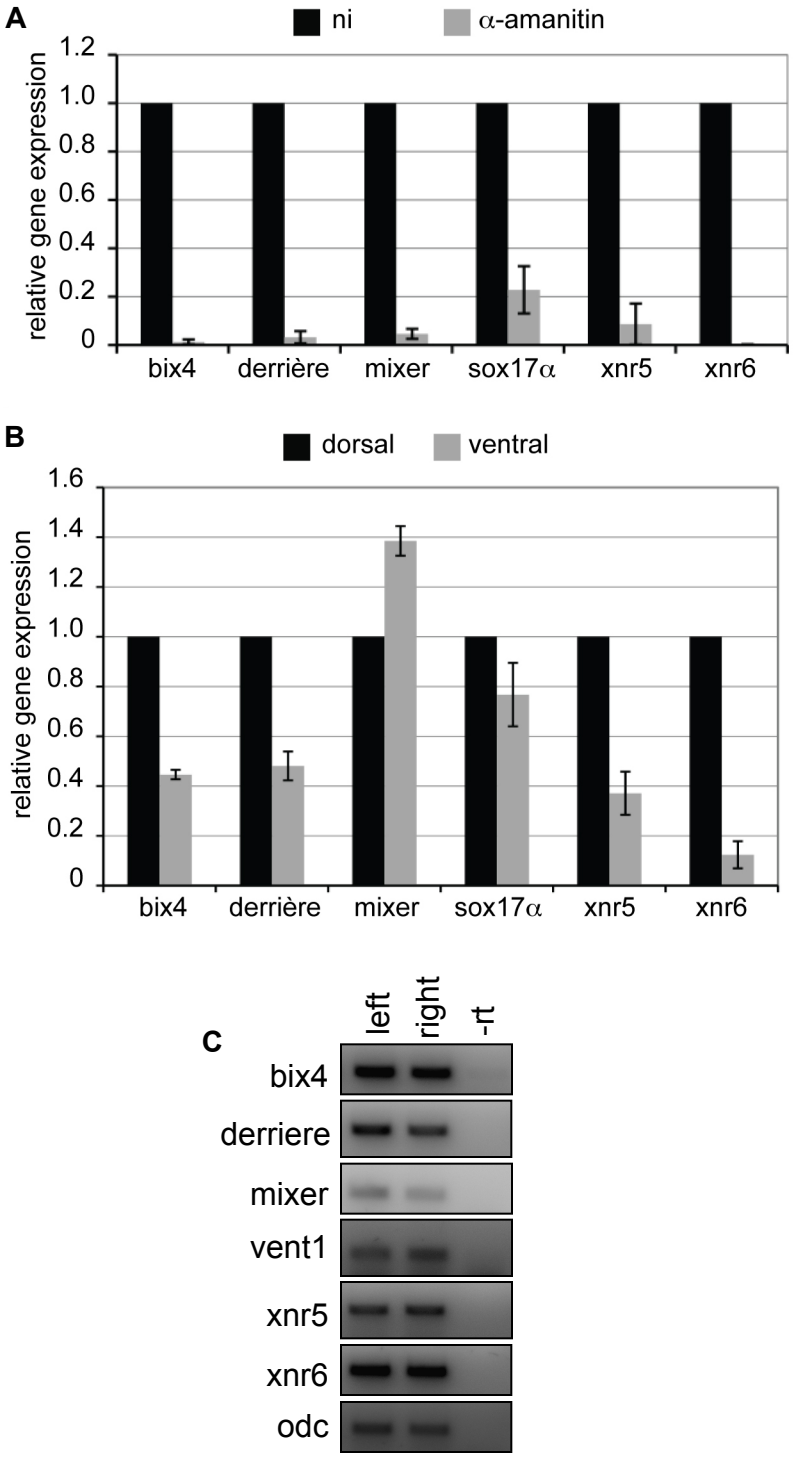

A
ni
α-amanitin
relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0
bix4
derrière
mixer
sox17α
xnr5
xnr6
B
dorsal
ventral
relative gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
bix4
derrière
mixer
sox17α
xnr5
xnr6
C
left
right
-rt
bix4
derriere
mixer
vent1
xnr5
xnr6
odc

Figure 2.3: Temporal analysis of Xnr family gene expression.

Embryos were cultured until the indicated time and harvested. RT-PCR was performed as described (Fig. 2.1) for the six Xnr family genes along with *odc* as a loading control.

Figure 2.3

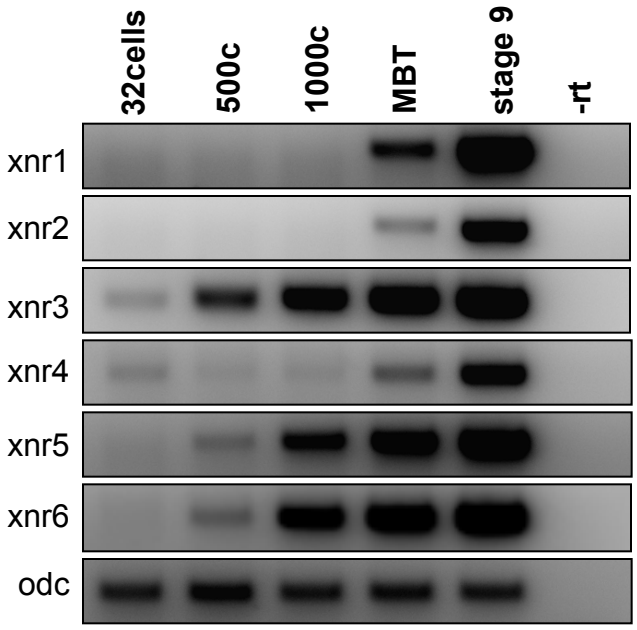
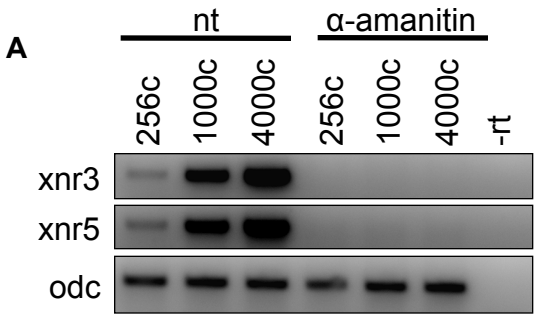

32cells
500c
1000c
MBT
stage 9
-rt
xnr1
xnr2
xnr3
xnr4
xnr5
xnr6
odc

Figure 2.4: preMBT expression of *xnr3* requires RNA Pol II.

(A) α-amanitin (100pg/embryo) was injected into both cells at the 2-cell stage. Injected and non-injected controls were harvested at the indicated stages for RT-PCR analysis. **(B)** The PCR product from a 1000-cell *xnr3* PCR was digested with RsaI for 1 hour then subject to gel electrophoresis along with undigested PCR product. The enzyme cleaved the product to produce the predicted products of 146 bp and 92 bp. MW = molecular weight marker.

Figure 2.4


A
nt
α-amanitin
256c
1000c
4000c
256c
1000c
4000c
-rt
xnr3
xnr5
odc

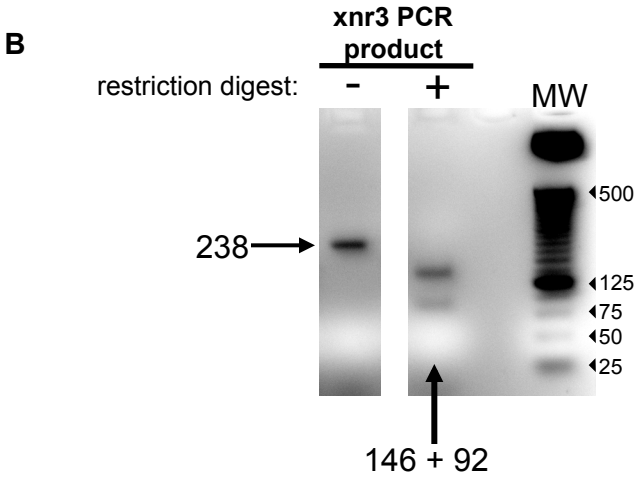

B
xnr3 PCR product
restriction digest:
-
+
MW
500
125
75
50
25
238
146 + 92

Figure 2.5: Analysis of various previously published *xnr3* primer sets.

(A) Schematic of *xnr3* cDNA showing the location and size of PCR products produced from each primer set. a = (Ding et al., 1998); b = (Agius et al., 2000); c = (Kofron et al., 2001); d = (Kofron, Matt et al., 2004); e = (Nelson and Gumbiner, 1999).

(B) Embryos were harvested at the indicated stages and RT-PCR was performed for *xnr3* using the indicated primer set, in concert with a standard curve of the indicated copy number of *xnr3* plasmid.

Figure 2.5

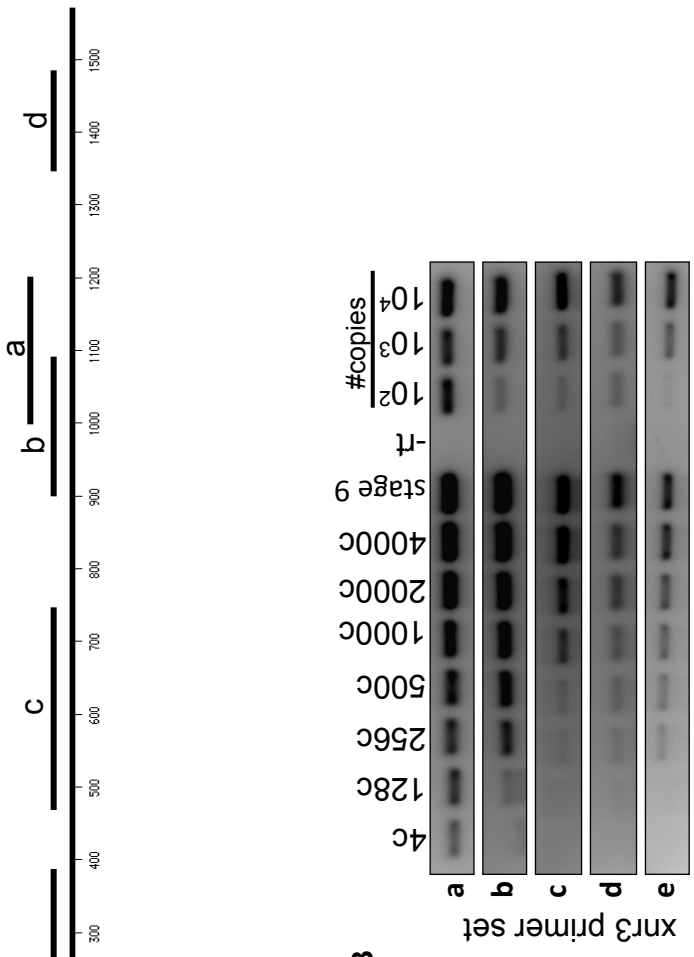

A
xnr3
e
c
b
a
d
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
B
xnr3 primer set
a
b
c
d
e
4c
128c
256c
500c
1000c
2000c
4000c
stage 9
-rt
#copies
10²
10³
10⁴

Figure 2.6: Quantitative analysis of *xnr3* expression.

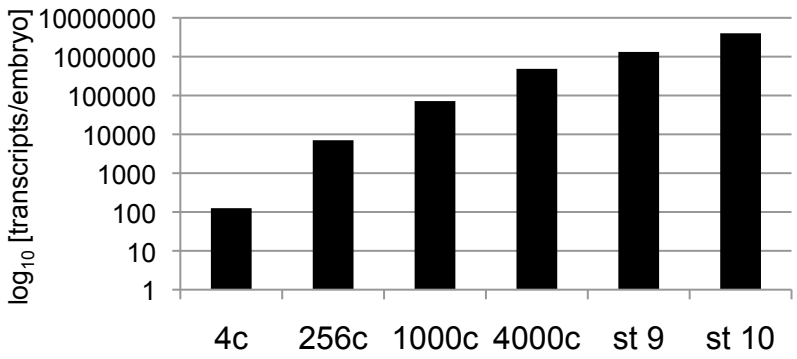
(A) Quantitative RT-PCR was performed on staged embryos and the number of *xnr3* transcripts/embryo was determined as described in Materials and Methods. Log_{10}[transcripts/embryo] was plotted. MBT = 4000c; -rt = no reverse transcriptase.

(B) List of transcripts per embryo at various stages for endodermin (*edd*), *xnr3*, *xnr5* and *xnr6*. Transcript number calculated as in A.

(C) Inhibition of RNA Pol II leads to a >98% decrease in preMBT expression of *xnr3*. α-amanitin (100pg/embryo) was injected into both cells at the 2-cell stage. Injected and non-injected controls were harvested at the 1000-cell stage (preMBT) for qRT-PCR analysis. The results were normalized to expression in the absence of α-amanitin (ni) for each gene. ni, not injected.

Figure 2.6

A



B

	edd	xnr3	xnr5	xnr6
4 c	202	125	175	2
256 c	188	7045	2333	121
1000 c	131	71808	12994	2595
4000 c	152	485491	43060	13519
st 9	4236	1323865	85076	46501
st 10	398918	3999754	143521	87999

C

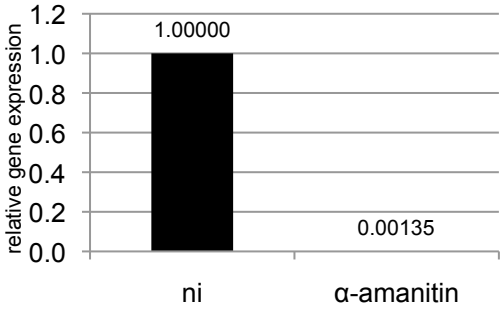


Figure 2.7: preMBT expression of *xnr3* is dorsally localized and requires β-catenin.

(A) Depletion of β-catenin leads to a >98% decrease in preMBT expression of *xnr3*. Antisense morpholino oligonucleotide (MO) targeting *ß-catenin* (40ng/embryo) was injected laterally into two cells at the 2-cell stage. Embryos were harvested for *xnr3* qRT-PCR at the 1000-cell stage. ni, not injected. Error bars represent standard error of the mean for 2 or more experiments.

(B) Two ventral cells at the 4-cell stage were injected with rhodamine dextran as a lineage tracer, dissected into dorsal and ventral halves at the 256-cell stage, then harvested at the 1000-cell stage for qRT-PCR. Error bars represent standard error of the mean for 2 or more experiments.

(C) In situ hybridization was performed on staged embryos for *xnr3* transcripts. 1000c stage and 4000 cell stage, dorsal view, animal pole to the top. Stage 10, vegetal view, dorsal to the top. Experiment performed by summer intern and high school student Anna-Claire Sienna.

Figure 2.7

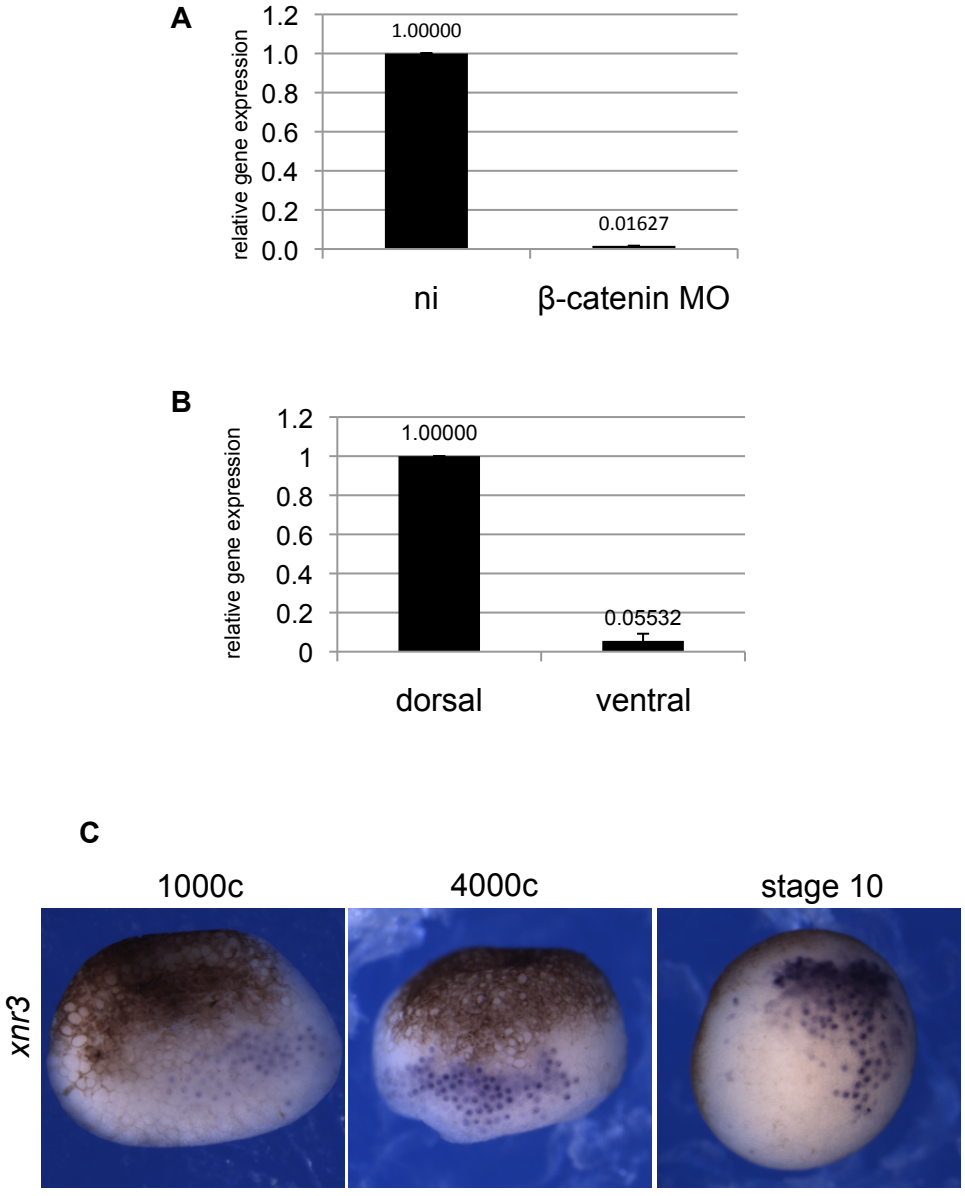
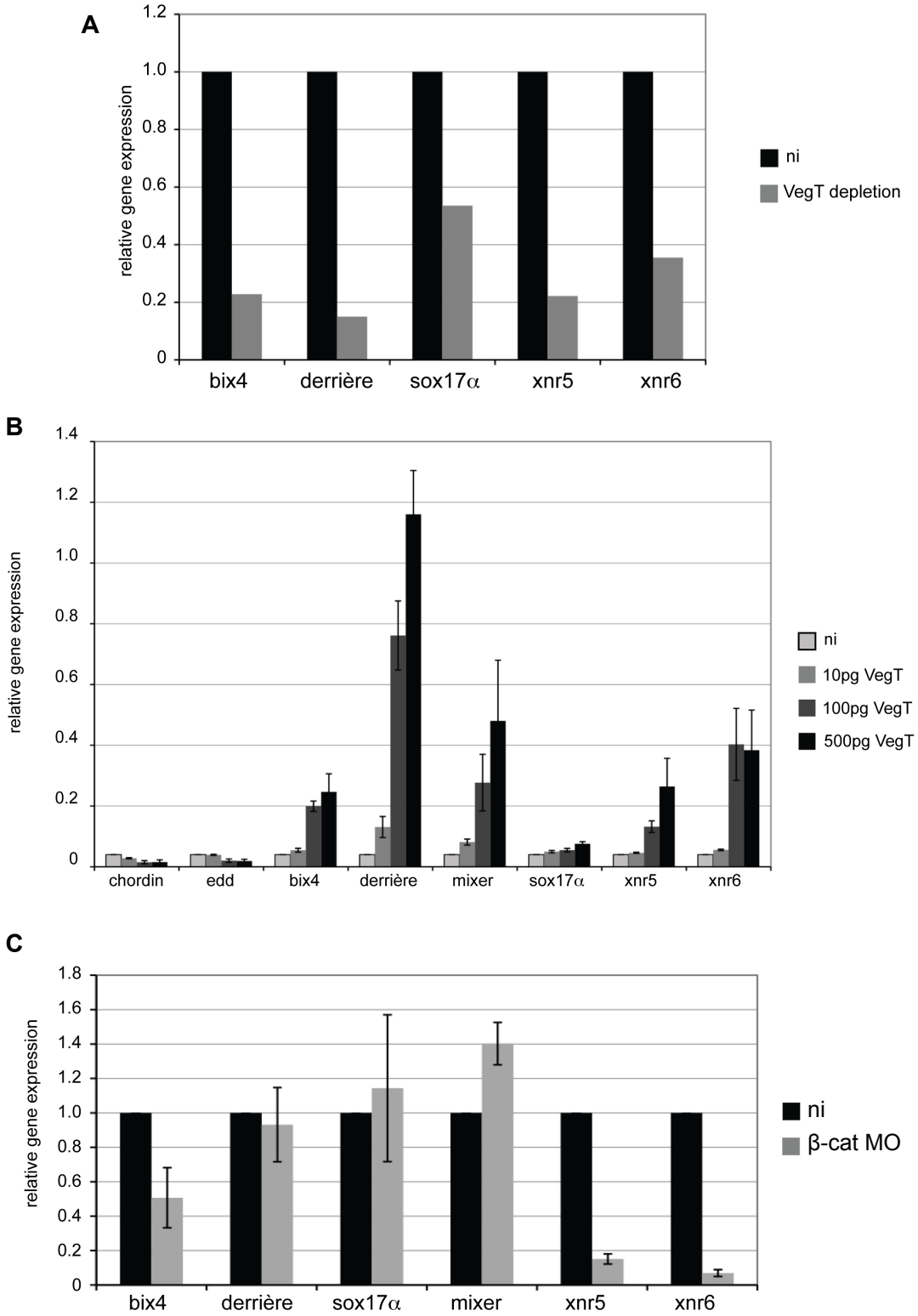

A
relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.00000
0.01627
ni
β-catenin MO
B
relative gene expression
1.2
1
0.8
0.6
0.4
0.2
0
1.00000
0.05532
dorsal
ventral
C
1000c
4000c
stage 10
xnr3

Figure 2.8: VegT is required for preMBT transcription.

(A) VegT mRNA was depleted in oocytes with 5ng antisense phosphorthiotate oligos and embryos were recovered using the host-transfer method. Control and VegT depleted embryos were collected at the 1000-cell stage for qRT-PCR. Maternal depletion and cDNA synthesis was performed by Jing Yang, Ohio State University.
(B) VegT mRNA was injected into the animal pole at the 1-cell stage. Animal caps were excised at the 256-cell stage and harvested for qRT-PCR at the 1000-cell stage. Error bars represent standard error of the mean for 2 or more experiments.
(C) Antisense morpholino oligonucleotide (MO) targeting *ß-catenin* (40ng/embryo) was injected laterally into two cells at the 2-cell stage. Embryos were harvested for qRT-PCR at the 1000-cell stage. ni, not injected. Error bars represent standard error of the mean for 2 or more experiments.

Figure 2.8


A
relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0
bix4
derrière
sox17α
xnr5
xnr6
ni
VegT depletion
B
relative gene expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
chordin
edd
bix4
derrière
mixer
sox17α
xnr5
xnr6
ni
10pg VegT
100pg VegT
500pg VegT
C
relative gene expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
bix4
derrière
sox17α
mixer
xnr5
xnr6
ni
β-cat MO

CHAPTER 3:

Nodal signaling is activated by preMBT transcription and is required during preMBT stages for development in *Xenopus laevis*[2]

[2] Work in this chapter was performed in collaboration with Guillaume Luxardi and Laurent Kodjabachian, CNRS-Universite de la Mediterranee, Marseille, France.

SUMMARY

In this chapter I examine the role of *xnr5* and *xnr6* in the early embryo. I find that activation of Nodal signaling begins before the MBT, and is dorsally localized. Nodal activity is dependent on transcription before the MBT, specifically on the preMBT expression of *xnr5* and *xnr6*. Loss of *xnr5* and *xnr6* together results in dorsal-anterior defects, shortened body axis, and a reduction of mesendodermal markers, as previously published. Importantly, inhibition of Nodal signaling leads to similar defects. However, Nodal signaling must be inhibited before the MBT to disrupt mesoderm induction and dorsal-anterior structures, suggesting that preMBT Nodal signaling is important for mesendoderm specification and patterning. Interestingly, Smad2 can rescue mesendodermal gene expression only if activated before but not after the MBT, supporting the conclusion that preMBT transcription and early Nodal activity is required for mesendoderm specification.

INTRODUCTION

Regulated transcription prior to the midblastula transition is a conserved phenomenon throughout the animal kingdom suggesting that it may have an important role in development, however few studies have addressed the role of preMBT transcripts in cell fate specification and patterning. In *Drosophila* the maternal transcription factor Zelda is required for preMBT gene activation, including genes required for cellularization (Liang et al., 2008). Embryos mutant for Zelda do not express these genes and fail to complete cellularization, an event that normally occurs at the MBT. While these observations support that preMBT transcription is critical during early embryogenesis, they do not address the significance of preMBT transcription of specific mRNAs and do

not address whether preMBT transcription plays a role in patterning or cell fate specification during early development.

Three of the eight *Xenopus* preMBT genes described in Chapter 2 are ligands in the Nodal/TGFβ signaling pathway (*derrière*, *xnr5*, and *xnr6*). The Nodal signaling pathway is important for endoderm and mesoderm specification and patterning in the embryo (Shen, 2007). The Nodal ligands transduce their signals through the TGFβ type I & II receptors. This results in the phosphorylation of Smad2 or Smad3, and the association of Smad2/3 with Smad4 (Massague et al., 2005). The activated P-Smad2/3-Smad4 complex enters the nucleus and targets genes for activation by binding to the transcriptional co-factor FoxH1 or *Mixer*. Targets of Nodal signaling include several organizer genes such as *goosecoid*, *chordin* and *xlim*, as well as other pan-mesodermal genes such as *xbra* and other Nodals (De Robertis, 2009).

Although Nodal function is essential in the early embryo, little is known about the contribution of individual Xnrs to mesoderm induction. Knock-down of Xnr gene activity through the use of antisense morpholino oligonucleotides (MOs) has been difficult, particularly for *xnr5* which has been duplicated several times resulting in 15 copies, all of which have slightly divergent 5'UTRs, making effective MO design difficult (Takahashi et al., 2006). Recent successful *xnr5* and *xnr6* loss of function experiments determined that these genes are important for the early induction of mesoderm in the embryo, as well as the activation of other Nodal ligands including *xnr1* and *xnr2*. *xnr1* and *xnr2* in turn are responsible for the expression of movement effector genes and for gastrulation movements (Luxardi et al., 2010).

Phosphorylation of Smad2, a hallmark of Nodal signaling, is first seen after the MBT on the dorsal side of the embryo in a region corresponding to *xnr5* and *xnr6* expression, an observation that correlates well with the role of *xnr5* and *xnr6* as zygotic initiators of

mesoderm formation (Agius et al., 2000; Lee, M. A. et al., 2001; Schohl and Fagotto, 2002). As gastrula stages approach, the location of P-Smad2 gradually shifts from the dorsal side to the ventral side of the embryo. The dorsal-vegetal region of the embryo is exposed to Nodal signaling earlier than other parts of the embryo, thus gradual loss of P-Smad2 in this region by the onset of gastrulation is most likely due to negative auto-regulation of Nodal signaling by Nodal targets. Additionally, activation of the pathway by other Nodals in the medial and ventral regions of the vegetal pole results in an increased P-Smad2 signal in these areas during late blastula and early gastrula stages. Several groups have suggested that the early activation of Nodal signaling in the dorsal vegetal area of the embryo is crucial for the establishment of the organizer (Agius et al., 2000; De Robertis et al., 2000; Faure et al., 2000; Lee, M. A. et al., 2001). Indeed, Nodal signaling activates many organizer genes in this area, and their early activation at the MBT may be required to establish and preserve a zone of dorsal character before the ventralizing effects of BMP signaling are manifested just after the MBT.

A long standing question about mesoderm induction in the frog has been when the mesoderm inducing signal is first generated. By using Nieuwkoop recombinant assays, some groups have established that the vegetal mass produces a mesoderm-inducing signal before the MBT, as early as stage 6 (Jones and Woodland, 1986; Ding et al., 1998). Other groups have found that the early vegetal pole cannot produce a mesoderm-inducing signal before the MBT (stage 8.5), and that Nodal signaling is not detected until after the MBT at stage 9 (Faure et al., 2000; Lee, M. A. et al., 2001; Saka et al., 2007). By using enhanced methods of detection, I show that endogenous Nodal signaling can be detected before the MBT and that its activity is dependent on preMBT transcription. Furthermore, loss of *xnr5* and *xnr6* transcripts before the MBT results in a reduction of Nodal activity at the MBT. Knock down of *xnr5* and *xnr6* activity also results

in a shortened body axis, loss of dorsal anterior structures, and reduction in mesendodermal markers. Additionally, constitutive inhibition of Nodal signaling has a very similar and very severe effect on the embryo; however selective inhibition of postMBT Nodal signaling results only in a shortened body axis, with well formed dorsal and anterior structures. These data suggest that preMBT expression of *xnr5* and *xnr6* is essential for the early activation of Nodal signaling and the development and patterning of mesoderm.

MATERIALS and METHODS

Xenopus Embryo Manipulations

Embryos were obtained by in vitro fertilization, cultured, and injected as described (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryos were maintained at 23ºC and the intervals between the first 8 cell divisions were closely monitored for each clutch of eggs. The timing of the 10^{th} through the 12^{th} cleavage divisions was predicted based on the length of the earlier cell cycles (approximately 22 minutes at 23ºC) and consistency in staging between different clutches was ensured by photographing embryos at each stage. Animal cap explants were excised and cultured as described (Sive et al., 2007). Dorsal-ventral dissections were performed by first injecting ventral blastomeres (determined by pigmentation) at the 4-cell stage with fluorescent dextran and then manually dissecting 256-cell stage embryos with a hair knife under epifluoresence. SB-505124 (Sigma, St. Louis, MO, USA) was dissolved in DMSO (100mM) and for embryo treatment was added to injection buffer (0.5X Marc's modified ringers solution (MMR) with 3% Ficoll (Sigma, St. Louis, MO, USA) and 50µg/ml gentamycin) to a final concentration of 200µM in glass culture dishes.

mRNA Injection

Synthetic capped mRNAs were produced with the mMessage mMachine kit (Ambion, Austin, TX, USA) using SP6 polymerase. Smad2 was amplified by PCR and cloned into pCS2-GRZ. The *xnr5* open reading frame was PCR-amplified from pNRRX-*xnr5* (Takahashi et al., 2000) and cloned into pCS2 to make pCS2-*xnr5*. pCS2-Smad2-GR, pCS2-*Xnr1* (Sampath et al., 1997) and pCS2-*xnr5* were linearized with Not1.

Morpholinos and VegT Maternal Depletion

Antisense morpholino oligonucleotides (MOs) targeting *xnr5* and *xnr6* (Luxardi et al., 2010) were purchased from GeneTools LLC (Philomath, OR, USA). Reduction in biological activity of *xnr5* and *xnr6* upon MO injection and rescue using recombinant Nodal protein were described previously (Luxardi et al., 2010).

RT-PCR and qRT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on staged embryos as described (Blythe et al., 2010), except that non-radioactive nucleotides were used and PCR products were separated via gel electrophoresis on a 2.5% agarose gel and visualized with ethidium bromide. cDNA was synthesized using random primers (Yang et al., 2002). Primers are described in Table 3.0 and Table 2.1.

Quantitative RT-PCR (qRT-PCR) was performed as described (Blythe et al., 2009). For each experiment, individual samples were analyzed in triplicate. Unless otherwise noted, data were analyzed by first normalizing to ODC loading control, and then calculating fold change compared to the indicated control condition using the ΔΔC(t) method (reviewed

in (Taneyhill and Adams, 2008)). Embryonic cDNA was synthesized as described above, using random primers.

Immunoprecipitation and Western Blot

For immunoprecipitation, 100-200 embryos per group were lysed and immunoprecipitated with 25 µL polyclonal anti-Smad2 antibody (#sc-6200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 25 µL polyclonal anti-Wnt1 antibody (negative control; #sc-6280, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described (Blythe et al., 2010). Western blots were performed by transferring proteins separated by SDS-PAGE to nitrocellulose, blocking membranes at room temperature for 1 hour in 5% non-fat milk in TBS-T (tris buffered saline with 0.1% tween-20) and immunoblotted with anti-P-Smad2/3 (1:500; #3101, Cell Signaling, Danvers, MA, USA), or anti-Smad2/3 (1:2000; #07-408, Upstate, Billerica, MA, USA) overnight at 4°C in blocking solution. HRP-linked secondary antibody and ECL Plus (GE Healthcare, Bucks, UK) were used to visualize bands. Image J software was used to quantify bands.

In situ hybridization and Antibody Staining

Whole-mount *in situ* hybridization (WISH) with digoxygenin labeled probes (Roche, Indianapolis, IN, USA) was carried out as described (Marchal et al., 2009) using the following probes: *sox17*α (Hudson et al., 1997), *Xbra* (Smith et al., 1991), *goosecoid* (Yasuo and Lemaire, 2001), *mixer* (Henry and Melton, 1998), and *xnr1* (Jones et al., 1995).

RESULTS

xnr6 mRNA is spliced prior to the MBT

If preMBT *xnr5* or *xnr6* transcripts function as Nodal ligands in the early embryo, the RNA must be processed and translated into protein. As low level read through by RNAP II can generate unspliced mRNAs without generating mature mRNAs, it is formally possible that the preMBT transcripts we detect represent unspliced mRNAs (Hargreaves et al., 2009). In the absence of available antibodies for *xnr5* or *xnr6*, we determined if preMBT *xnr6* mRNA was spliced to its mature form, or if the transcripts we detect represent a non-spliced mRNA precursor (Fig 3.0). We designed primers flanking the two introns of *xnr6*, exon 1-2 junction primers and exon 2-3 junction primers (Fig 3.0 A). These primers discriminate between spliced and non-spliced forms of transcript depending on the size of the PCR product generated by RT-PCR. RT-PCR on a timecourse of embryos revealed that the preMBT *xnr6* transcripts detected do indeed reflect mature, spliced mRNA, supporting that these transcripts are most likely translated to proteins in the early embryo (Fig 3.0 B, C).

preMBT expression of *xnr5* and *xnr6* activates Nodal signaling

Expression of *xnr5* and *xnr6* in the embryo prior to large-scale ZGA suggests that these Nodal ligands could play an early role in mesendoderm induction. One of the hallmarks of activated Nodal signaling is the presence of phosphorylated Smad2 (Shen, 2007). In previous reports, Smad2 phosphorylation was first detected by immunoblotting in late blastulae (stage 9 to 9.5) (Faure et al., 2000; Lee, M. A. et al., 2001) and by immunofluoresence at stage 8 (1000-cell stage) (Schohl and Fagotto, 2002). As *xnr5* and *xnr6* are present prior to the MBT and the Nodal pathway can be activated by

ectopic expression of activin prior to the MBT (Lee, M. A. et al., 2001), we tested whether *xnr5* could induce Smad2 phosphorylation prior to the MBT. We injected *xnr5* mRNA into embryos and performed Western blot analysis on embryos collected at various time points using an antibody specific for Smad2 phosphorylated at C-terminal serines (P-Smad2). Indeed, expression of exogenous *xnr5* mRNA induced P-Smad2 as early as the 128-cell stage (stage 7); in contrast, an equivalent amount of *xnr1* mRNA did not induce P-Smad2 until after the MBT (Fig. 3.1 A). Thus the Nodal pathway can function well before MBT and is sensitive to relatively low concentrations of *xnr5* mRNA.

As *xnr5* and *xnr6* are expressed before the MBT, we hypothesized that the endogenous Nodal pathway may also be activated by these ligands before the MBT. To enhance detection of phosphorylated Smad2, we immunoprecipitated Smad2 from *Xenopus* embryos at different stages of development and then detected P-Smad2 by Western blot. Using this approach, we observed a weak P-Smad2 signal as early as the 1000-cell stage and a stronger signal at the 2000-cell stage (Fig. 3.1 B), indicating that Nodal signaling is initiated at least by the 1000 to 2000-cell stage, consistent with Schohl and Fagotto, who detected P-Smad2 by immunofluoresence at stage 8 (1000-cell stage)(Schohl and Fagotto, 2002). Furthermore, as preMBT *xnr5* and *xnr6* expression is restricted to dorsal blastomeres, we tested whether P-Smad2 is asymmetrically distributed in early embryos. Embryos were dissected into dorsal and ventral halves, and P-Smad2 was detected by Western blot. Consistent with the early dorsal distribution of *xnr5* and *xnr6*, the phosphorylation of Smad2 at the MBT is dorsally enriched (Fig. 3.1 C); this observation is also consistent with the immunofluoresence data reported by Schohl and Fagotto (Schohl and Fagotto, 2002), as well as immunostaining and Western blot data in stage 9 embryos reported by others (Faure et al., 2000; Lee, M. A. et al., 2001).

To determine if early Nodal activation requires zygotic gene expression, we decided to analyze P-Smad2 levels in the absence of zygotic transcription. Interestingly, injection of early embryos with the pol-II inhibitor α-amanitin blocked endogenous phosphorylation of Smad2 at the MBT (Fig. 3.2 A), indicating that activation of Nodal signaling requires preMBT transcription. Smad2 phosphorylation can be restored in the presence of α-amanitin by injection of *xnr5* mRNA, demonstrating that, aside from the ligand, other essential components of the pathway do not require new transcription before the MBT (Fig. 3.2 A). The findings that Nodal signaling requires new transcription before the MBT, and is localized in the same region as preMBT *xnr5* and *xnr6* expression, led us to ask if *xnr5* and *xnr6* are required for this early signaling activity. Targeting *xnr5* and *xnr6* with antisense morpholino oligonucleotides (previously characterized in (Luxardi et al., 2010)) reduced Smad2 phosphorylation at the MBT by 52% to 74% compared to control MO levels, showing that preMBT expression of *xnr5* and *xnr6* is required for early activation of Nodal signaling (Fig. 3.2 B). These observations strongly support that Nodal signaling is activated before the MBT and depends on preMBT transcription of Nodal ligands.

xnr5 **and** ***xnr6*** **depletion results in convergent extension defects and reduction of mesendodermal markers**

Consistent with previously published experiments, *xnr5* and *xnr6* MO co-injection in Figure 3.2 B resulted in severe patterning defects in the early embryo (Luxardi et al., 2010). Embryos injected with both *xnr5* and *xnr6* MO exhibited a shortened axis (previously shown to be the result of convergent extension defects) and a loss of dorsal-anterior structures including the eye, head, brachial arches and dorsal fin, as well as a reduction in head and muscle markers (Fig. 3.3). The total body length of the late tailbud

embryo was reduced significantly as was the mesoderm marker xbra and the endoderm marker *sox17*α at early gastrula stage (Figs. 3.4 A, B and C). These results together confirm previous findings that *xnr5* and *xnr6* are likely required for mesendodermal induction, dorsal-ventral patterning and embryo elongation (Luxardi et al., 2010).

Inhibition of Nodal signaling before MBT disrupts mesendoderm induction

Although morpholino oligonucleotides offer a powerful approach to knock down gene expression, injection of MOs is only feasible through early cleavage stages. To test the requirement for Nodal signaling at multiple stages from early cleavage through late blastula, we used the small molecule inhibitors SB505124 (SB5) and SB431542, which inhibit Nodal signaling by selectively inhibiting the kinase activity of activin receptor-like kinases ALK4, 5, and 7 (Watabe et al., 2003; DaCosta Byfield et al., 2004). These drugs reduce phosphorylation of Smad2 in *Xenopus* embryos (Luxardi et al., 2010) (Figure 3.5 A), without affecting Smad1/5/8 (Inman et al., 2002; DaCosta Byfield et al., 2004) (data not shown). When SB5 was applied at the 4-cell stage, embryos failed to gastrulate and by tailbud stage the larvae were severely deformed, lacking axial structures (Fig. 3.5 B) and showing a severe reduction in all mesendodermal markers analyzed (Fig. 3.5 B, C). Similar morphological defects were observed with SB431542 (data not shown). However, in contrast to the marked effects observed when the drug was applied at the 4-cell stage, the severity of the morphological defects declined as the inhibitor was applied at later time points. Although these embryos showed delayed gastrulation and a failure of the blastopore to close completely, consistent with a previous report (Luxardi et al., 2010), the tadpoles nevertheless developed elongated anterior-posterior axes, clear dorsal-ventral polarity, and anterior structures including eyes, brain vesicles, cement gland, dorsal fin, and tail, structures that were not observed when embryos were treated

at the 4-cell stage (Fig. 3.5 B). Similarly, the expression of mesendodermal markers was most sensitive to inhibitor added at the 4-cell stage, as analyzed by qPCR (Fig. 3.5 C) and by in situ hybridization (Fig. 3.6) on embryos harvested at the onset of gastrulation (stage 10).

To confirm that these results reflect inactivation of Nodal signaling soon after addition of SB5 to media, we tested how rapidly SB5 inhibits P-Smad2 in vivo after addition to the culture medium (Fig. 3.7). 50pg *xnr5* mRNA was injected at the 1-cell stage to induce preMBT accumulation of P-Smad2. Injected embryos were cultured in control media or media supplemented with SB5, beginning at the 128-cell stage. Embryos were collected at various times points after addition of SB5 and analyzed for P-Smad2. When *xnr5* mRNA is injected, P-Smad2 begins to accumulate at the 128-cell stage. SB5 inhibits Smad2 phosphorylation within two cell cycles, and existing P-Smad2 decreases gradually in the presence of SB5, presumably through dephosphorylation or protein turn-over (Fig. 3.7). These results indicate that Nodal signaling is inhibited soon after drug addition.

These data suggest that Nodal signaling before the MBT plays an important role in mesendodermal development. To test this further, we used an inducible form of Smad2 fused to the glucocorticoid receptor ligand-binding domain (Smad2-GR) to rescue the mesendodermal gene expression in the presence of the Nodal inhibitor. Embryos injected with Smad2-GR mRNA were treated with SB5 from the 4-cell stage. Smad2-GR was then activated by addition of dexamethasone (dex) for a 160-minute window either before or after the MBT. Mesendodermal gene expression was then measured at stage 10. SB5 treatment alone greatly reduced mesendodermal gene expression (Fig. 3.8). Activation of Smad2 by addition of dex from the 4-cell to the 500-cell stage restored mesendodermal gene expression to similar levels as untreated

control embryos. Activation of Smad2 after the MBT was less effective in restoring mesendodermal gene expression (Fig. 3.8). This shows that preMBT Nodal signaling contributes significantly to the restoration of mesendodermal gene expression.

DISCUSSION

Nodal signaling is essential in vertebrates for the establishment and patterning of mesoderm and endoderm. In this work, we find that the maternal T-box transcription factor VegT is required for the preMBT transcription of a group of genes involved in mesendoderm development, including the Nodal genes *xnr5* and *xnr6*; we also show that Nodal signaling in *Xenopus* embryos is initiated before the MBT and that preMBT transcription of *xnr5* and *xnr6* is essential for this activity. These findings establish a critical function for zygotic transcription before the MBT in *Xenopus* embryos.

In previous work, Smad2 phosphorylation and nuclear localization were first detected in stage 9 blastulae (Faure et al., 2000; Lee et al., 2001; Saka et al., 2007), whereas others detected P-Smad2 by stage 8/1000-cell stage (Schohl and Fagotto, 2002). Similar to Faure et al and Lee et al, we show that activation of Nodal signaling requires zygotic transcription. Furthermore, we show that *xnr5* and *xnr6* transcribed before the MBT are required for early activation of this pathway (Figure 3.1), as they are for phosphorylation of Smad2 in the gastrula stage (Luxardi et al., 2010). Detection of phosphorylated Smad2 in the embryo is limited by the sensitivity of the antibody and the detection method; by using a different antibody and immunoprecipitating Smad2, we have enhanced the sensitivity to detect Smad2 phosphorylation as early as the 2000-cell stage, and it is likely that Smad2 is phosphorylated earlier. Schohl and Fagotto speculated that early P-Smad2 is activated by a maternal TGFβ/Nodal signal (Schohl and Fagotto, 2002), but their findings are also consistent with the zygotic expression and

activity of *xnr5* and *xnr6* before the MBT reported here, in addition to a contribution from Vg1 (Birsoy et al., 2006), a maternal TGFβ-related ligand (Weeks and Melton, 1987).

The importance of preMBT transcription mediated by VegT may reflect the fact that VegT activates a gene regulatory network that is required for establishment of the primary germ layers, one of the earliest cell fate decisions in vertebrate embryos. While the expression of mesoderm and endoderm markers begins, for the most part, after the MBT, earlier Nodal signaling, along with the positive autoregulation through *xnr5* and *xnr6* reported here, may be required to initiate the postMBT phase of Nodal signaling (*xnr1, 2, and 4*) and mesendoderm induction. This conclusion is consistent with prior work showing that Nodal/TGFβ signaling is required for the initiation of *xnr1*, *2*, and *4* expression after the MBT (Agius et al., 2000; Luxardi et al., 2010). Agius et al proposed Vg1 as a candidate molecule for this signal, but their data are also consistent with the preMBT activity of *xnr5* and *xnr6* reported here.

Furthermore, Jones and Woodland reported that mesoderm-inducing signals are present by stage 6 to 6.5, well before the MBT (Jones and Woodland, 1986). Similarly, Ding and colleagues detected a mesoderm-inducing signal between the 16-cell and 128-cell stage (Ding et al., 1998), and Wylie et al reported a weak ventral mesoderm inducing activity present before the MBT, although they also reported that dorsal mesoderm inducing activity was not detectable (in Nieuwkoop recombinants) until after the MBT (Wylie et al., 1996). Jones and Woodland concluded that the mesoderm-inducing signal they detected was most likely encoded by a maternal gene. However, subsequent molecular work showed that VegT-dependent zygotic expression of Nodal-like ligands is required for mesendodermal induction (Zhang et al., 1998; Kofron et al., 1999; Agius et al., 2000). Our data, taken together with evidence for the role of maternal Vg1 in mesoderm induction (Birsoy et al., 2006), could help to reconcile these findings,

as we have shown that the zygotic transcription of *xnr5* and *xnr6* begins by stage 6.5 (64 cells)(Yang et al., 2002) and their preMBT expression is required for Smad2 phosphorylation (Figure 3.1) and mesoderm induction. Our findings are compatible with the observation that maternal Vg1 also contributes to Smad2 phosphorylation, induction of mesendodermal genes, and development of dorsal mesodermal structures (Birsoy et al., 2006).

xnrs 1, 2, 4, 5 and *6* are expressed initially at higher levels in dorsal cells, and Smad2 phosphorylation is also initially enriched in dorsal cells (Lee et al., 2001)(Figure 3.1). Several groups have proposed that dorsal Nodal expression is required for development of the Spemann organizer and expression of organizer specific genes (reviewed in (Whitman, 2001)). Furthermore, Agius et al proposed that *VegT* and a maternal Nodal-like signal such as *Vg1* may synergize with the dorsal specifying activity of the Wnt/β-catenin pathway to generate a zygotic gradient of xnrs in blastula-stage endoderm (Agius et al., 2000). Similarly, Lee et al proposed that the initial dorsal enrichment of Smad2 phosphorylation arises through cooperation between VegT and β-catenin (Lee et al., 2001). Our data strongly support these ideas, as we show that VegT and β-catenin directly activate preMBT expression of *xnr5* and *xnr6* in a spatially restricted manner in the dorsal vegetal cells known as the Nieuwkoop center and that preMBT transcription is also required for the dorsally enriched phosphorylation of Smad2. These observations are also consistent with a two-step model for mesoderm induction, wherein a low level of Nodal signaling initiates mesoderm induction before the MBT, contributing to a higher level after the MBT (Kimelman and Griffin, 1998; Yasuo and Lemaire, 1999; Takahashi et al., 2000), except that we propose that preMBT zygotic Nodals contribute significantly to the initial mesoderm-inducing signal.

PreMBT expression of Nodals and the VegT-induced transcription factors *bix4*, *mixer*, and *sox17α* may serve to segregate distinct functions of Nodal signaling in the early embryo. Previous work showed that early signaling through *xnr5* and *xnr6* contributes to mesendoderm induction whereas later Nodal signaling through *xnr1* and *xnr2* activates movement-effector genes involved in gastrulation (Luxardi et al., 2010). Similarly, the Wnt/β-catenin pathway has distinct functions during the blastula stage, and these must be separated temporally in the early embryo (Blythe et al., 2010). Dorsal specifying genes such as *siamois* and *twin* are first expressed after the MBT; however, these genes require Wnt/β-catenin signaling during early cleavage stages and become insensitive to the pathway by the MBT (Christian and Moon, 1993; Darken and Wilson, 2001). In this context, dorsal Wnt target genes are primed for expression at the MBT by β-catenin dependent chromatin modifications conferred on promoters during earlier stages of development (Blythe et al., 2010). Thus maternal Wnt signaling marks dorsal organizer genes before MBT for expression at the MBT. Through this mechanism, organizer genes are protected from postMBT Wnt signaling, which is required for ventral and posterior patterning.

Taken together, these findings demonstrate at least two preMBT functions for maternal transcription factors: Maternal Wnt/β-catenin signaling is required before the MBT to preset organizer genes for later expression at the MBT, whereas VegT directly activates preMBT transcription of genes required for mesendodermal induction. Both mechanisms confer critical temporal control on pathways that set up the earliest cell fate decisions in the embryo, dorsal-ventral specification and primary germ layer induction.

Interestingly, these two mechanisms overlap in the regulation of *xnr5* and *xnr6*, as the initial, early expression of these genes in the Nieuwkoop center depends on both Wnt/β-catenin and VegT function. How VegT targets escape global transcriptional

repression in the preMBT embryo remains unclear and is a topic for future study. Whereas Wnt target genes are poised for expression before the MBT, VegT may recruit transcription elongation factors to complete transcription of these target genes. However, VegT by itself is clearly not sufficient to confer preMBT transcription, as at least two direct targets of VegT are not transcribed until after the MBT; perhaps activation by VegT in conjunction with loss of repression by an unidentified factor is required to confer gene expression before the MBT. Future work will investigate what distinguishes pre and postMBT VegT targets and investigate whether additional maternal transcription factors regulate preMBT gene expression.

FIGURES AND FIGURE LEGENDS

Table 3.0: RT-PCR Primers

	Sequence	Source
xbra fwd	5'- GGATCGTTATCACCTCTG -3'	Smith et al., 1991
xbra rev	5'-GTGTAGTCTGTAGCAGCA -3'	
cerberus fwd	5'- ATTCACTTAACAGCAGAGGT -3'	Klein Lab
cerberus rev	5'- CTTCTAGAACCATTGTAAGC -3'	
dkk fwd	5'- ACAAGTACCAACCTCTGGATGC -3'	Glinka et al., 1998
dkk rev	5'- ACAGGGACACAAATTCCGTTGC -3'	
eomes fwd	5'- CCATCCAAACCTCCCACC -3'	Glinka et al., 1998
eomes rev	5' –CTTCTCTTACATGCACCCG -3'	

Please see Table 2.1 for additional RT-PCR primer sequences

Figure 3.0: *xnr6* is spliced before the MBT

(A) Schematic of the *xnr6* gene showing three exons (black) and two introns (blue and green). Right and left arrows represent the approximate location of forward and reverse PCR primers, respectively. The first set in blue amplifies the exon 1-2 junction (blue) and the second set amplifies the exon 2-3 junction (green). Not drawn to scale. Primers pairs: exon 1-2 junction, fwd: 5'- ACA CCC TGC TCT TCA GGA ATC CG -3', rev: 5'- TGT GCC TTC TGG TGC CAG GGA -3'; exon 2-3 junction, fwd: 5'- TTC CCT GGC ACC AGA AGG CAC -3', rev: 5'- TCA GTT GCA TCC ACA CTC TTC AAC AA -3'.

(B) RT-PCR for the *xnr6* exon 1-2 junction. Two exposures of the same gel are shown (light, dark). Embryos were collected at the indicated time, PCR was performed on cDNA and products were separated by size with gel electrophoresis. Genomic *xnr6* (*xnr6* gene) was used as a positive control for amplification of *xnr6* intron 1. Spliced and unspliced product sizes are indicated on the far right.

(C) RT-PCR for the *xnr6* exon 2-3 junction. Experiment same as in (B) using primer set to amplify exon 2-3 junction instead.

Figure 3.0

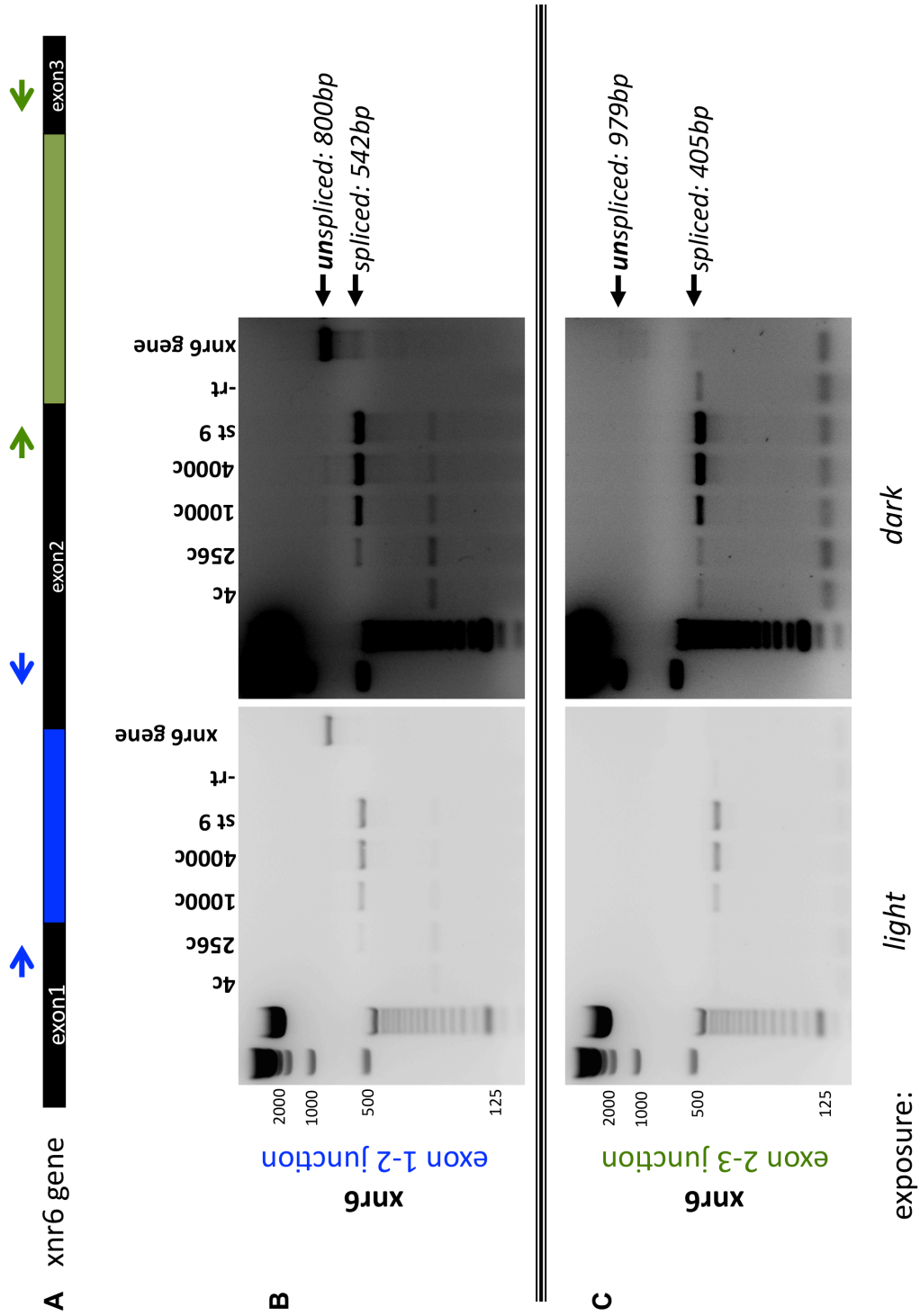

A xnr6 gene
exon1
exon2
exon3
B
xnr6
exon 1-2 junction
unspliced: 800bp
spliced: 542bp
xnr6 gene
-rt
st 9
4000c
1000c
256c
4c
2000
1000
500
125
C
xnr6
exon 2-3 junction
unspliced: 979bp
spliced: 405bp
exposure:
light
dark

Figure 3.1: **Nodal signaling is dorsally localized at the MBT and can be detected before the MBT.**

(A) *xnr5* mRNA (5pg) or *xnr1* mRNA (5pg) was injected vegetally into 1-cell embryos. Embryos were collected at the indicated stages and lysates were analyzed by Western blot for P-Smad2 and Smad2/3.

(B) Non-injected embryos were collected at the indicated stages and subjected to immunoprecipitation with an antibody for Smad2 or for Wnt1 as a negative control (control), then immunoblotted with antibodies for P-Smad2 or Smad2/3. Band intensity was determined by quantifying pixel density from a scanned film using Image J; the level of P-Smad2 was then normalized to total Smad2/3.

(C) Embryos were injected ventrally at the 4-cell stage with rhodamine dextran, dissected into dorsal and ventral halves at the 256-cell stage, then harvested at the 4000-cell stage for IP and Western analysis as in B. we, whole embryo.

Figure 3.1

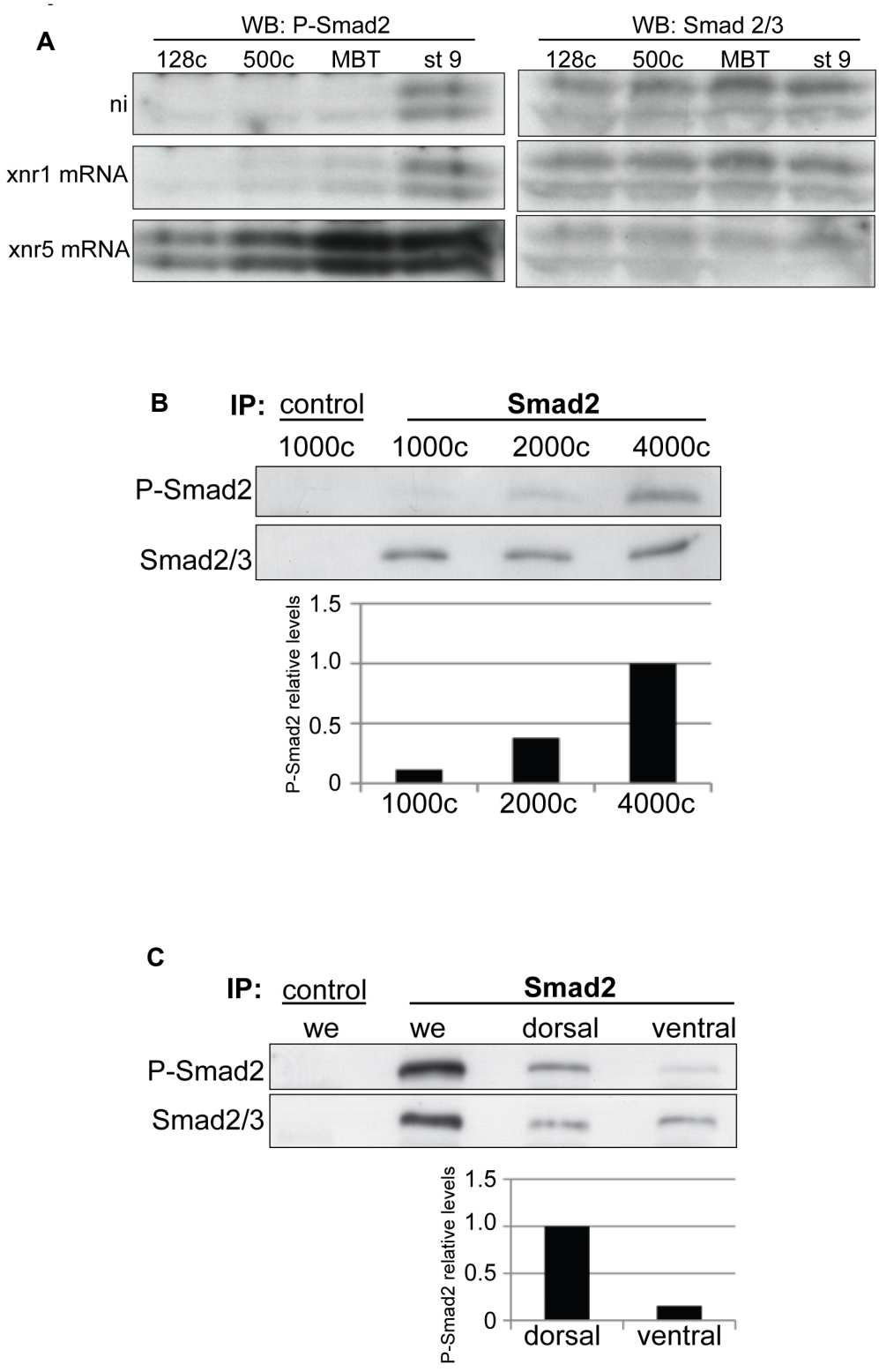
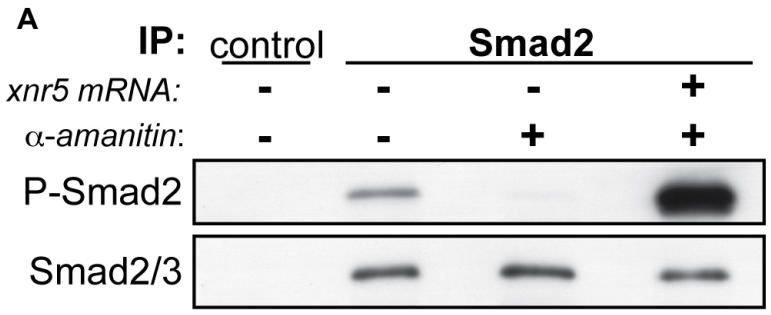

A
WB: P-Smad2
WB: Smad 2/3
128c
500c
MBT
st 9
ni
xnr1 mRNA
xnr5 mRNA
B
IP: control
Smad2
1000c
1000c
2000c
4000c
P-Smad2
Smad2/3
P-Smad2 relative levels
1.5
1.0
0.5
0
1000c
2000c
4000c
C
IP: control
Smad2
we
we
dorsal
ventral
P-Smad2
Smad2/3
P-Smad2 relative levels
1.5
1.0
0.5
0
dorsal
ventral

Figure 3.2: Nodal signaling at the MBT requires preMBT transcription of *xnr5* and *xnr6*.

(A) 1-cell embryos were injected laterally with 100pg of the pol-II inhibitor α-amanitin alone or together with 5pg *xnr5* mRNA, then collected for IP and immunoblot at the 4000-cell stage (MBT) as in Fig 3.1 B.

(B) *xnr5* and *xnr6* translation was inhibited by co-injection in all cells of 4-cell embryos of two sets of antisense morpholino oligonucleotides (a MOs 15ng each, b MOs 15ng each, a+b MOs 7.5ng each, control MO 30ng) and embryos were collected at the 4000-cell stage for IP and immunoblot as in Fig 3.1 B. ni, not injected; CMO, control morpholino oligonucleotide. MO-injected embryos were provided by Guillaume Luxardi and Laurent Kodjabachian.

Figure 3.2


A
IP:
control
Smad2
xnr5 mRNA:
- - - +
α-amanitin:
- - + +
P-Smad2
Smad2/3

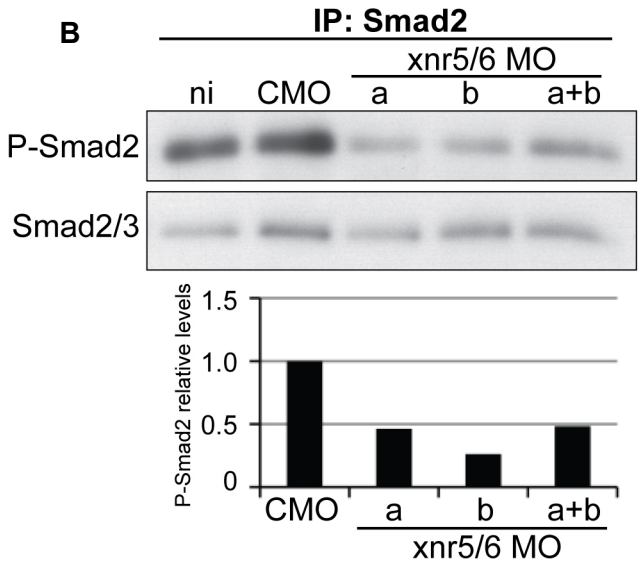

B
IP: Smad2
xnr5/6 MO
ni
CMO
a
b
a+b
P-Smad2
Smad2/3
P-Smad2 relative levels
1.5
1.0
0.5
0
CMO
a
b
a+b
xnr5/6 MO

Figure 3.3: Phenotype of *xnr5* and *xnr6* MO injected embryos.

Embryos injected as in Figure 3.2 B were analyzed for phenotype at late tailbud stage. Whole embryo phenotypes are shown, as well as in situ analysis for otx2 or 12.101 to show the presence of head and muscle tissues, respectively. The graph below indicates the distribution of phenotypes (A-D) in embryos injected with *xnr5* + *xnr6* MO type a, or MO type b. MO, morpholino oligonucleotide. Experiments in this figure were performed by Guillaume Luxardi and Laurent Kodjabachian.

Figure 3.3

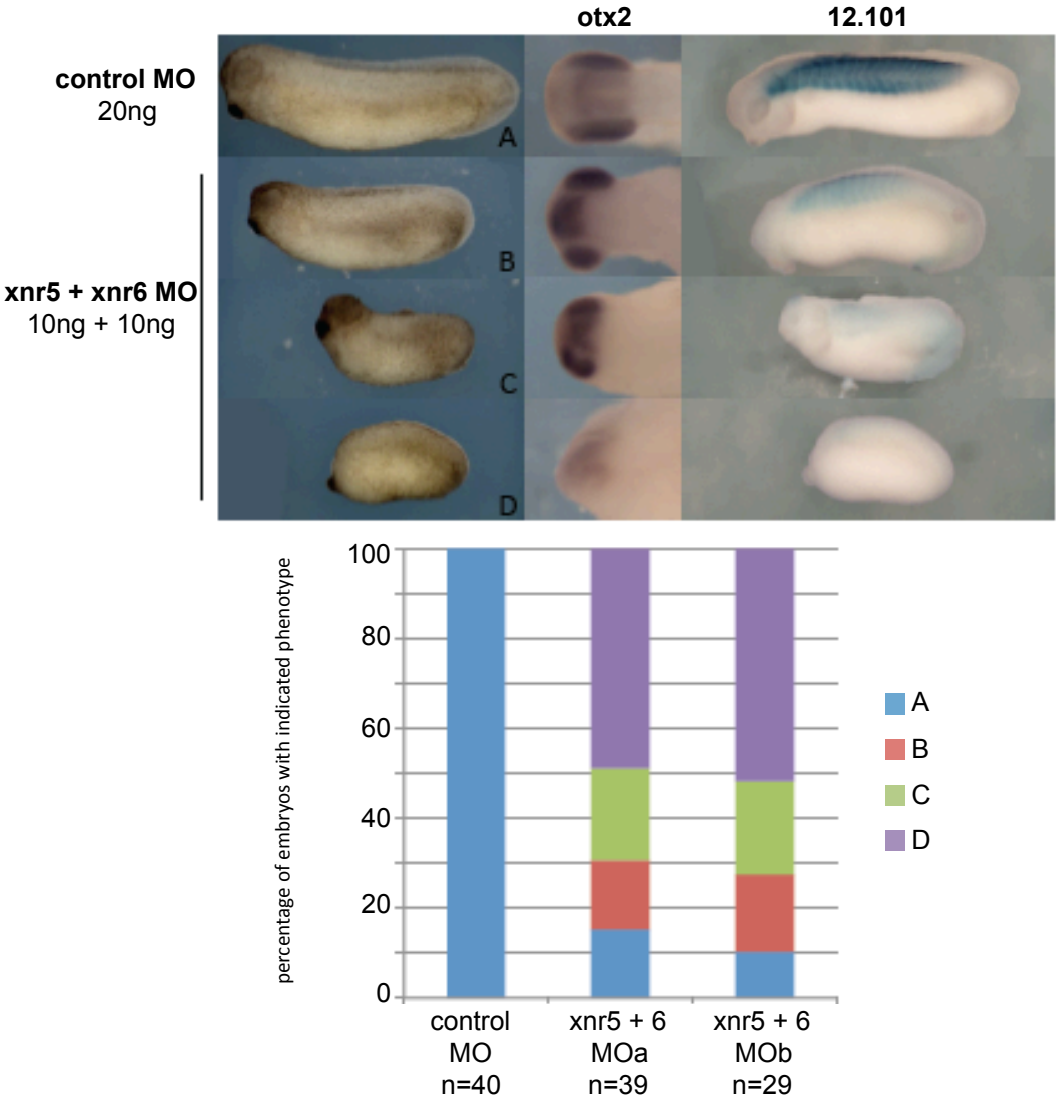

otx2
12.101
control MO
20ng
xnr5 + xnr6 MO
10ng + 10ng
A
B
C
D
percentage of embryos with indicated phenotype
100
80
60
40
20
0
control MO n=40
xnr5 + 6 MOa n=39
xnr5 + 6 MOb n=29
A
B
C
D

Figure 3.4: *xnr5* and *xnr6* MO reduce embryo length and mesendodermal gene expression

(A) Phenotypes of embryos injected with *xnr5* + *xnr6* MOa or MOb.

(B) Length of embryo was measured from anterior end to posterior end for embryos in each group in (A). Average length in pixels was plotted using a box-plot diagram.

(C) Expression of the mesodermal gene xbra and the endodermal gene *sox17*α was assessed using qRT-PCR at stage 10. Both genes were reduced in all *xnr5* + 6 MO-injected groups. MO, morpholino oligonucleotide. Experiments performed by Guillaume Luxardi and Laurent Kodjabachian.

Figure 3.4

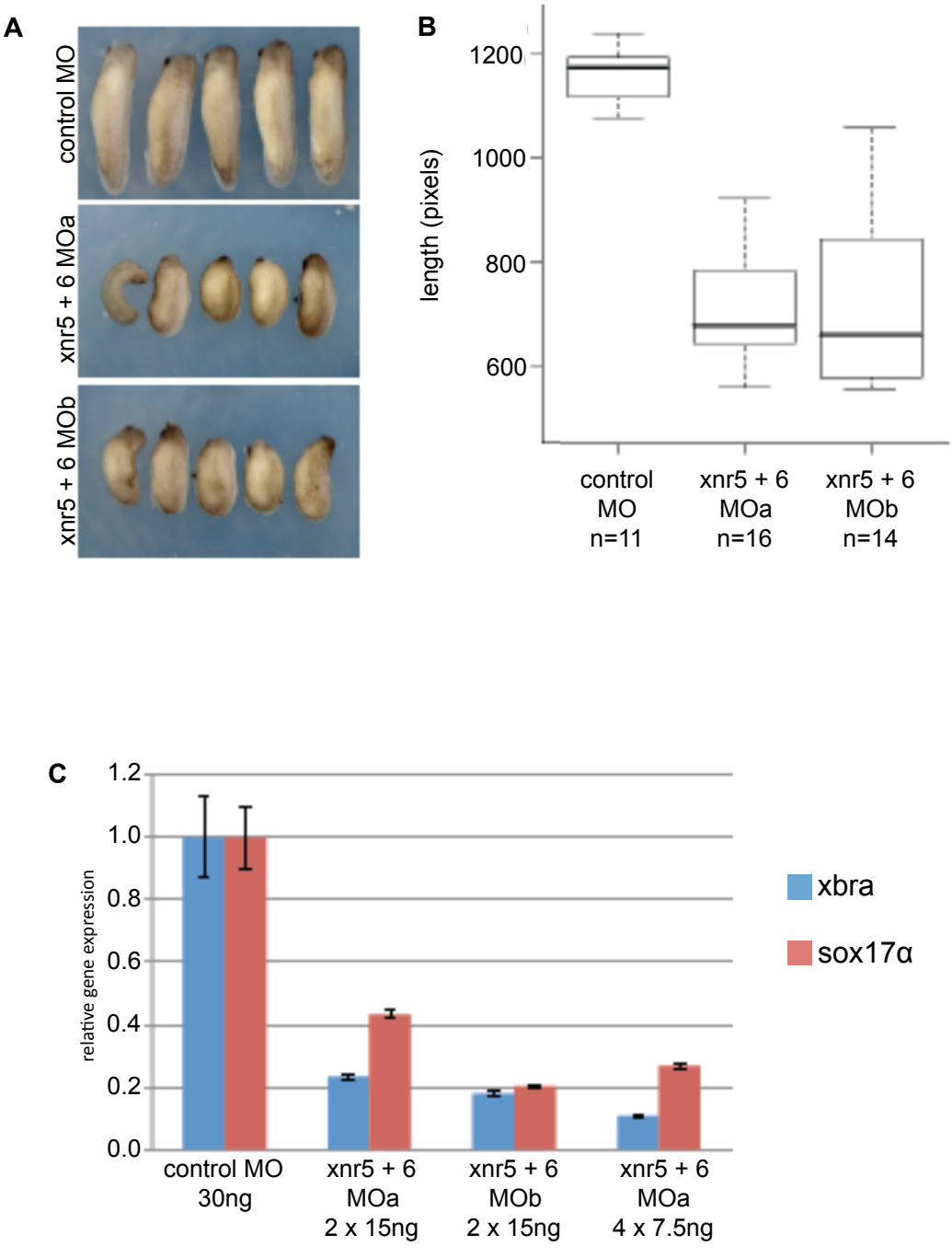

A
control MO
xnr5 + 6 MOa
xnr5 + 6 MOb
B
length (pixels)
1200
1000
800
600
control MO n=11
xnr5 + 6 MOa n=16
xnr5 + 6 MOb n=14
C
relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control MO 30ng
xnr5 + 6 MOa 2 x 15ng
xnr5 + 6 MOb 2 x 15ng
xnr5 + 6 MOa 4 x 7.5ng
xbra
sox17α

Figure 3.5: preMBT Nodal signaling is required for mesendoderm induction.

(A) Embryos were treated with 200 μM SB505142 (SB5) or control medium from the 4-cell stage to stage 10 and harvested for Western blot for P-Smad2 or Smad2/3.

(B) Embryos were treated with SB5 at the indicated stages, cultured until stage 40, and then analyzed for morphology. Frequency of phenotype is indicated in the lower right corner.

(C) Embryos from B were collected at stage 10 for qRT-PCR for endoderm and mesoderm markers. Signal intensity is normalized to the no treatment control values for each marker.

Figure 3.5

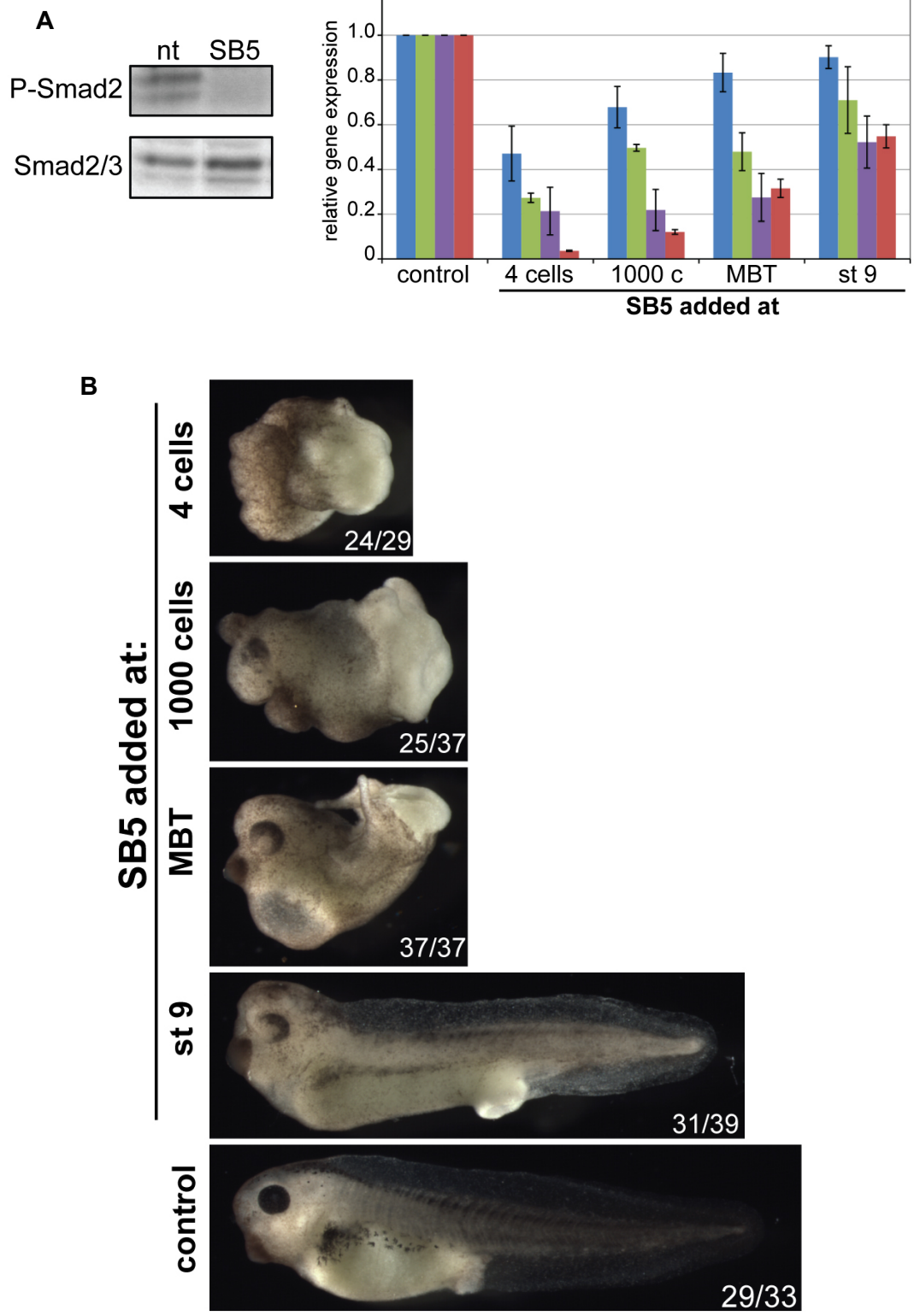

A
nt
SB5
P-Smad2
Smad2/3
C
edd
sox17α
xnr1
xbra
relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0
control
4 cells
1000 c
MBT
st 9
SB5 added at
B
SB5 added at:
4 cells
1000 cells
MBT
st 9
control
24/29
25/37
37/37
31/39
29/33

Figure 3.6: preMBT Nodal signaling is required for mesendoderm induction. Embryos were treated with SB5 (200 μM) at the indicated stages, cultured until stage 10 and analyzed by in situ hybridization for mesendodermal gene expression. Experiment performed by Guillaume Luxardi and Laurent Kodjabachian.

Figure 3.6

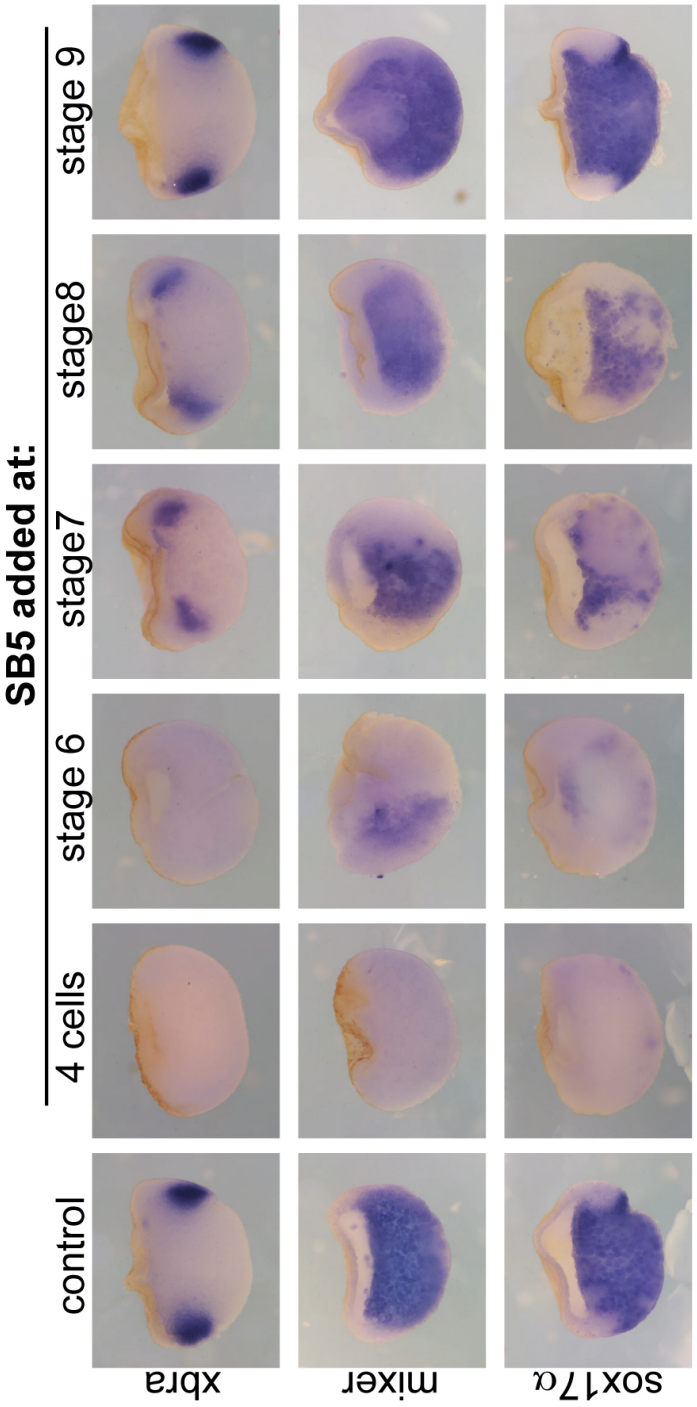

SB5 added at:
control
4 cells
stage 6
stage7
stage8
stage 9
xbra
mixer
sox17α

Figure 3.7: SB5 quickly prevents accumulation of P-Smad2 in the early embryo. Embryos were injected with 50pg *xnr5* mRNA unilaterally at the 1-cell stage to induce preMBT accumulation of P-Smad2. Injected embryos were then cultured in control media or media supplemented with SB5 beginning at the 128-cell stage. Embryos were collected at the indicated times and analyzed for P-Smad2 or total Smad2/3 by Western Blot.

Figure 3.7

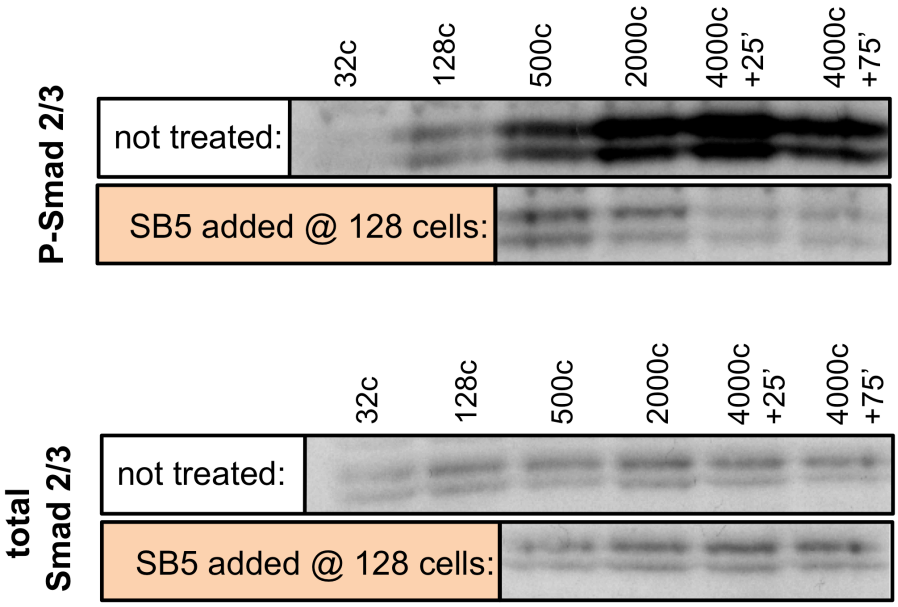
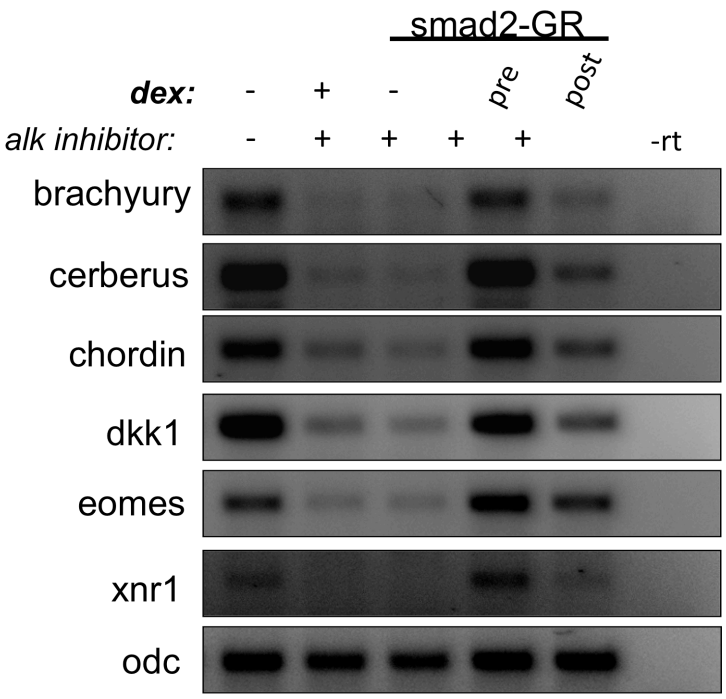

P-Smad 2/3
32c
128c
500c
2000c
4000c +25'
4000c +75'
not treated:
SB5 added @ 128 cells:
total Smad 2/3
32c
128c
500c
2000c
4000c +25'
4000c +75'
not treated:
SB5 added @ 128 cells:

Figure 3.8: preMBT Smad2 activity is required to rescue normal levels of mesendodermal gene expression in SB5 treated embryos.

Embryos were injected vegetal-laterally with Smad2-GR at the 1-cell stage and then treated with SB5 at the 4-cell stage. Smad2-GR was activated by addition of dexamethasone (dex) for a 160-minute window before the MBT (4-cells to 500-cells) or after the MBT (MBT to stage 10); embryos were collected for RT-PCR at stage 10.25. nt, not treated.

Figure 3.8


smad2-GR
dex: - + - pre post
alk inhibitor: - + + + + -rt
brachyury
cerberus
chordin
dkk1
eomes
xnr1
odc

CHAPTER 4: DISCUSSION

General conclusions from this work

In this thesis I identify several zygotic mesendodermal determinants that are expressed before the MBT. I also show that preMBT expression of most of these genes requires the maternal T-box transcription factor VegT. VegT is also sufficient for the preMBT expression of these genes in ectodermal tissue explants. Three of these genes are TGFβ ligands, and are preferentially expressed in the dorsal vegetal region of the early embryo. I also identify the divergent Nodal-related ligand and organizer gene *xnr3* as a preMBT gene. Additionally, I show that Nodal signaling is active at the onset of the MBT (stage 8.5) and that preMBT transcription is required for this early activation. Furthermore, inhibition of early Nodal signaling results in malformation of the embryo, including a shortening of the anterior-posterior axis, loss of dorsal-anterior structures and a concomitant decrease in mesendodermal markers. Reduction of *xnr5* and *xnr6* function with antisense MOs has the same effect on the embryo. In contrast, inhibition of only late Nodal signaling (from the MBT onwards) results in an embryo with a clearly defined anterior-posterior axis, normal dorsal-anterior structures, and robust expression of a subset mesendodermal genes; the embryo still exhibits defects in lengthening of the anterior posterior axis and reduced expression of other mesendodermal genes. Collectively, these results show for the first time that preMBT transcription is essential for early development, and specifically required for one of the earliest developmental decisions, establishment and patterning of the primary germ layers.

Discussion and Future Directions

Regulation of preMBT transcription

The discovery of preMBT transcription is relatively recent and as such its regulation has not been studied in great detail. In *Drosophila*, genome-wide analysis revealed that the promoters of most preMBT genes contain one or more related heptameric sequences termed the TAGteam sites (ten Bosch et al., 2006). Addition or removal of TAGteam sites to a promoter advances or delays expression, respectively, demonstrating that the timing of preMBT gene activation in *Drosophila* is in part regulated by conserved sequences in the 5' region of preMBT genes. Prior to this finding, the prevailing model to explain preMBT gene silencing and the regulated activation of genes at the MBT was titration of a repressor due to increase in nuclear-to-cytoplasmic ratio caused by successive rounds of nuclear division. However the discovery of TAGteam sequences suggested that temporal regulation of genome activation was controlled by a transcriptional activator and not simply by passive titration of repressive factors. Indeed, shortly after discovery of TAGteam sequences was the finding that the transcriptional activator Zelda regulates activation of preMBT genes through binding to these sequences (Liang et al., 2008).

Prior to this thesis, the only preMBT genes described in *Xenopus* were *xnR5* and *xnr6*. The preMBT expression of these genes is dependent on β-catenin, however no other β-catenin target genes analyzed are activated before the MBT. In this thesis, I show that *xnr5*, *xnr6*, *sox17α*, *mixer*, *bix4*, *derrière* and *vent1*, all targets of the maternal T-box transcription factor VegT, are expressed before the MBT. Although VegT is a common activator of these genes, only *xnr5*, *xnr6*, *sox17α*, *bix4*, *derrière* and *vent1* are direct targets of VegT, presumably all containing T-box binding sites in their promoter

regions through which VegT mediates its response. *mixer* cannot be induced by VegT in the presence of cycloheximide, and therefore is not considered a direct target of VegT (Taverner et al., 2005). Although *xnr3* is robustly expressed before the MBT, its regulation by VegT is not well characterized. VegT cannot induce *xnr3* in ectodermal explants and VegT abrogates the ability of β-catenin to induce *xnr3* in ectodermal explant assays, suggesting it may serve as a negative regulator of *xnr3* (Rex et al., 2002), while other work shows that *xnr3* expression is significantly reduced in VegT depleted embryos, suggesting that VegT is required for activation of *xnr3* (Kofron et al., 1999; Xanthos et al., 2002). As discussed in Chapter 3, this likely reflects several levels of regulation of *xnr3* by VegT.

Although sequence for the *mixer* promoter region is not available for *X. laevis*, the *mixer* promoter in *X. tropicalis* genome contains three core T-box sequences (CACCA/T) within 1 kb of the transcription start site for *mixer*, including one 18 basepairs upstream of the ATG (position 41921, GenBank AC151467) (Howard et al., 2007). Similarly, the *X. laevis xnr3* sequence contains a core T-box site 20 basepairs upstream of the ATG that is conserved in *X. tropicalis* (NCBI Reference Sequence, laevis NM_001085790.1; tropicalis NM_001112906.1). This demonstrates that core T-box sites are conserved in the 5' region of all preMBT genes identified, providing evidence that preMBT genes in Xenopus share common sequence elements in their promoter regions, a phenomenon also observed in *Drosophila*. Perhaps large-scale analysis of the 5' regions of zygotic genes for this motif will reveal new candidates for preMBT gene expression.

It will also be interesting to examine if VegT binds to these promoters before the MBT using chromatin immunoprecipitation (ChIP). Figure 2.0 demonstrates that myc-tagged VegT can bind to both the *xnr6* and the *xnr3* promoters in preMBT embryos. In

the absence of commercially available VegT antibodies, new antibody will need to be produced to demonstrate this interaction in non-injected preMBT embryos. Attempts in our lab to purify VegT protein for use in antibody production have not been successful due to solubility issues. Perhaps design of an expression construct containing a portion of the VegT sequence producing a smaller and more hydrophilic protein fragment will be a more efficient strategy to obtain antibody for use in ChIP assays. Not only will this allow our lab to confirm the association of VegT with preMBT targets, but it will also allow us to identify additional endogenous preMBT VegT targets through the use of ChIP-Seq. It will also be interesting to examine if VegT targets that are not expressed before the MBT are bound by VegT prior to the MBT, as such a finding might indicate an alternative preMBT function for VegT, as described below.

Our lab has previously shown that although β-catenin/TCF binds to the promoters of active genes before the MBT (*xnr5*, *xnr6*), it also associates with genes that are not active until the MBT, such as *siamois* (Blythe et al., 2010). In this context, β-catenin serves to recruit histone methyltransferase activity to target genes prior to the MBT, essentially priming these genes for activation at the MBT. Interestingly, a mammalian T-box gene, T-bet, has been shown to physically recruit H3K27-demethylase and H3K4-methylase activities, both chromatin marks (demethylated H3K27 and methylated H3K4) that are associated with active transcription (Lewis et al., 2007; Miller et al., 2008). Recruitment of these activities is modulated through the T-box domain of this protein, and upon further analysis the authors found that other T-box proteins including Brachyury and Eomesodermin also interact with the H3K27-demethylase activity, suggesting this function is conserved among T-box protein family members (Miller et al., 2008). Using histone methyltransferase assays, it will be interesting to determine if VegT also recruits this and other histone methyltransferase

activity to target promoters, facilitating the activation and perhaps poising of genes before the MBT. Along these lines, it will be interesting to see if, in addition to preMBT target genes, VegT associates with and modifies postMBT target genes during the preMBT period. In this case, further analysis will be needed to determine what discriminates preMBT from postMBT VegT targets, and how the former are active while the latter remain silent in the early embryo, as discussed below.

Unpublished ectodermal explant assays in our lab have shown that hormone-inducible VegT (VegT-GR) can induce postMBT expression of many mesendodermal markers including *goosecoid*, *cerberus*, *eomesodermin*, *frizbe* and *dickkopf* if VegT-GR is activated (by addition of dexamethasone) during the preMBT period. Activation of VegT at or after the MBT cannot induce these genes, suggesting that the ability of VegT to activate these genes is limited to a window of time during preMBT development. Further experiments showed that although the ability of VegT to activate these genes is dependent on preMBT VegT *activity*, it is not dependent on preMBT *transcription*: transient transcriptional inhibition during the preMBT period using actinomycin D was not able to prevent postMBT activation of these genes by VegT-GR. These observations are consistent with the possibility that VegT is required to recruit histone modifying factors (such as methyltransferase proteins) to these genes during the MBT so that they can be primed for activation after the MBT.

The ability of VegT to activate some, but not all targets, before the MBT suggests that another level of regulation is at play to discriminate pre- vs. postMBT targets. It is possible that postMBT VegT targets including chordin and endodermin have a repressive factor associated with their regulatory regions that is not present at preMBT VegT target genes. Release of this repressive factor by some mechanism after MBT would then allow VegT to activate postMBT transcription of these genes. Alternatively,

preMBT activation of VegT targets could require the presence of a secondary activating sequence and/or transcription factor that is not associated with postMBT VegT targets. Another possibility is that postMBT but not preMBT VegT targets require a secondary transcriptional activator that is only active after the MBT. In the embryo, any combination of these mechanisms is possible to ensure that some VegT targets are expressed before the MBT while others remain silent. In the absence of any candidates for additional repressors or activators, comparison of pre- and postMBT promoter sequences could reveal interesting patterns that distinguish preMBT from postMBT VegT targets.

Function of preMBT transcription in the embryo

Precise temporal regulation of the zygotic genome including preMBT large-scale silencing and precocious activation of specific genes is conserved in many organisms suggesting it might have an important developmental function. Pritchard and Shubiger proposed that prior to patterning events that begin upon large-scale gene activation (MBT), the zygotic genome must be potentiated for transcription. Thus, preMBT gene expression is merely reflective of "noise" as a result of the genome being prepared for activation. To illustrate this point, they use the following analogy:

> Before one can drive a car it is essential to first turn on the engine. The time between activating the engine and putting the car in drive is irrelevant, but the car will not drive unless the engine is engaged (Pritchard and Schubiger, 1996).

However several lines of evidence argue against this reasoning. Firstly, many preMBT genes in Drosophila are involved in dosage compensation, cellularization and pattern formation, all events that occur at the MBT (cycle 14). Work from the Rushlow and Cline laboratories has suggested that these genes must to be activated during the preMBT period to "set the stage" for these molecular processes upon large-scale genome activation (ten Bosch et al., 2006; Liang et al., 2008). According to their

hypothesis, failure to activate these genes prior to the MBT would result in a loss of coordinated gene response at the MBT. For example, it is essential that the embryo determine the number of X chromosomes each nucleus contains prior to the MBT so that the proper sex-specific gene profile can be activated at the MBT; X chromosome counting begins prior to cycle 12 and is regulated by the feminizing gene *sxl* (Cline and Meyer, 1996). Failure to activate the X chromosome counting machinery before cycle 12 would prevent the embryo from engaging dosage compensation machinery before the MBT, resulting in an imbalance of x-linked gene expression (Tracey et al., 2000). Although evidence suggesting the preMBT expression of sex-determination is required for later developmental processes is largely speculative, a more concrete example is that of cellularization genes. Loss of the global preMBT gene activator Zelda results in reduced expression of preMBT genes, including genes involved in cellularization. Thus Zelda mutants fail to cellularize at cycle 14 (MBT) (Liang et al., 2008), strongly supporting an essential role for preMBT transcription in cellularization. However, these data do not address a role for preMBT mRNAs in cell fate specification or patterning of the embryo.

Studying the requirement of preMBT transcription for later embryonic development is problematic because most transcriptional inhibitors available are irreversible, and therefore inhibit both pre- and postMBT transcription. When transcription is inhibited with α-amanitin, *Xenopus* embryos appear to divide normally during cleavage cycles, slow down their cell cycles at the MBT and develop until early gastrula stage 10 when they promptly expire; however, α-amanitin is not reversible and therefore this drug cannot be used to study loss of preMBT transcription alone. Transient inhibition of transcription with brief actinomycin-D exposure has been effective at inhibiting transcription for short periods of time, however actinomycin-D is also an

intercalation agent, and interferes with DNA replication and other cellular processes making it difficult to interpret experimental results in vivo. As a result of these difficulties, the best approach to study the contribution of preMBT transcription to later development is to target specific genes for knock-down using antisense morpholino oligonucleotides (MOs), then restore the gene function after MBT by injection of a plasmid rescue construct (that will not be transcribed until MBT due to preMBT silencing of exogenous DNA), or by injection and postMBT activation of a hormone-inducible rescue construct.

In this thesis I present compelling evidence that preMBT expression of *xnr5* and *xnr6* is required for early Nodal pathway activation, mesendodermal specification and possibly for patterning of the embryo; the implications of these findings will be discussed in the next section. I also show that several other genes involved in mesendoderm formation including *derrière*, *mixer*, *sox17*α, *bix4* and *vent1*, are expressed before the MBT. Although roles for many of these genes have been reported, it is not known if any of these genes function before the MBT. Since specification of the germ layers is one of the first patterning events that occur in the embryo, it is likely that these genes play an important role during early embryogenesis. For example, *mixer* is known to be an important endodermal determinant that establishes boundaries between regions of mesoderm and endoderm (Kofron, M. et al., 2004). The preMBT expression of *mixer* would allow the embryo to pre-set distinct regions of endoderm and mesoderm *before* large scale gene activation occurs; this would help compartmentalize expression of the appropriate genes at the ZGA into the appropriate mesodermal or endodermal territories.

Determining the function of these genes before the MBT will be an interesting future direction. Since several of these genes including *mixer*, *sox17*α, *bix4* and *vent1* are transcription factors a good way to study their preMBT function would be to knock

down expression with anti-sense MO, then rescue postMBT expression by using a rescue construct fused to the hormone-inducible glucocorticoid receptor (GR) activating domain. This approach would allow temporal control of the rescue construct activity by the addition of the GR-activating hormone dexamethasone. Since *derrière* is an extra-cellular ligand its activity is more difficult to control using a GR-coupled rescue construct. Its contribution to the preMBT embryo can be studied by anti-sense knock-down followed by injection with mRNA (expressed pre- and postMBT) or plasmid DNA (expressed postMBT only) constructs.

preMBT Nodal signaling

Although mesoderm induction in the *Xenopus* embryo has been studied for decades, the precise identity and timing of action of the initial mesoderm-inducing signal has remained a mystery. Many studies have suggested that the primary mesoderm-inducing signal is a Nodal-like or Activin-like ligand, however in the absence of knock-down data for individual ligands, it has been difficult to determine which of the eight ligands plays the principle role in mesoderm induction. In Chapter 3, I showed that activation of Nodal signaling begins as early as the 1000-cell stage. When analyzed at the MBT, early Nodal activity is dorsally localized and most importantly, almost entirely dependent on preMBT transcription. Further analysis revealed that preMBT expression of *xnr5* and *xnr6* is necessary for robust Nodal signaling at the MBT.

These findings are surprising since previous reports show accumulation of P-Smad2 by Western blot at stage 9 to 9.5, one to two hours after the MBT (Faure et al., 2000; Lee, M. A. et al., 2001). This discrepancy is likely due to the limits of detection in each assay: in this thesis I examine the temporal expression of P-Smad2 using a different antibody than previously published, and also by using Smad2

immunoprecipitation coupled with P-Smad2 Western blot to enhance the ability to detect low-levels of Nodal activity. Similarly, another group used biomolecular fluorescence complementation (BiFC) to determine that Smad2/Smad4 complexes do not enter the nucleus until after the MBT (Saka et al., 2007). However the authors were not able to confirm that nuclear entry of ectopic BiFC molecules can ever occur in rapidly cleaving preMBT cells, and they therefore lacked a crucial positive control for this assay. In contrast to these studies, another report shows the presence of P-Smad2 in preMBT embryos by whole-mount immunostaining, an observation which our results support (Schohl and Fagotto, 2002). Although the authors speculated that the early P-Smad2 signal was the result of maternal TGFβ signaling, we propose it was the result of preMBT *xnr5* and *xnr6* signaling activity

The work in Chapter 3 also established that preMBT Nodal signaling is important for mesendoderm specification and for development of dorsal-anterior structures in the embryo, demonstrating that preMBT transcription is crucial for important developmental processes. The finding that preMBT inhibition of Nodal signaling results in severe loss of mesendodermal gene expression and dorsal-anterior structures when compared to postMBT inhibition of Nodal signaling, although novel, is not entirely surprising. Recent work from the Kodjabachian lab suggested that early expression of *xnr5* and *xnr6* plays a distinct role from other Xnrs in the induction of mesoderm, although the preMBT contribution of these genes to development was not assessed in their study (Luxardi et al., 2010). Furthermore, work from two independent labs identified that vegetal pole explants can produce a mesoderm-inducing signal before the MBT, an observation that our data support (Jones and Woodland, 1986; Ding et al., 1998).

Although the experiments in Figure 3.5 do not rule out contribution to Nodal signaling from maternal Vg1 or Activin, the data in Figure 3.2 strongly suggest that the

majority of Nodal signaling observed at the MBT is the result of preMBT zygotic gene expression. Interestingly, when we examined the P-Smad2 signal detected two cell cycles before the MBT (Fig. 3.1 panel C), we found that it is not sensitive to transcriptional inhibition by α-amanitin, suggesting that maternal TGFβ contributes to a fraction of the P-Smad2 signal observed at the MBT. Alternatively, this early signal could be an artifact from interaction of the Western blot antibody with the immunoprecipitation antibody; performing a similar analysis on untreated verses Nodal inhibitor (SB5) treated embryos at this early stage would confirm if this signal is due to maternal Nodal signaling or not. Another interesting future direction will be to examine the association of Smad2 with Nodal target promoters in the preMBT embryo using chromatin immunoprecipitation (ChIP). This analysis could reveal the identity of some of the very first postMBT targets of preMBT Nodal signaling, as well as potential preMBT targets.

An additional function of *xnr5* and *xnr6* signaling before the MBT was recently revealed in work by our collaborators in the Kodjabachian laboratory. This work shows that preMBT Nodal signaling activates expression of *xnr5* and *xnr6* through a feed-forward mechanism, suggesting that Nodal signaling is also required for ensuring robust preMBT expression of *xnr5* and *xnr6*. Another future direction includes determining if this interaction is direct by examining if Smad2 physically interacts with the *xnr5* and *xnr6* promoters before the MBT using ChIP. It will also be interesting to test if inhibition of transcription prevents this interaction, as such an experiment would reveal the extent of maternal contribution to early Nodal signaling.

What advantage does preMBT Nodal signaling confer on the development of the embryo? The Rushlow and Cline groups proposed that preMBT expression of sex determination genes in *Drosophila* is important to ensure the proper sex-specific gene expression profile by the time large-scale zygotic gene expression begins at MBT (ten

Bosch et al., 2006; Liang et al., 2008). Similarly, in *Xenopus*, preMBT Nodal signaling might serve to preserve a region of dorsal mesodermal character, so that by the time large scale transcription begins at the MBT, a zone of active Nodal signaling has already been established. Several groups have suggested that early exposure to relatively high Nodal signals in the dorsal marginal region of the embryo is important for the induction of organizer genes and the patterning of dorsal mesoderm (Agius et al., 2000; Lee, M. A. et al., 2001). Along these lines, ectopic expression of *xnr5* can produce a partial secondary axis (Takahashi et al., 2000; Takahashi et al., 2006). The recent availability of antisense MOs for *xnr5* and *xnr6* will allow for a more detailed analysis of the function of these genes. By using both in situ hybridization and gene expression analysis, it will be very interesting to see to what extent dorsal mesodermal genes and the organizer is affected in *xnr5* and *xnr6* deficient embryos.

I propose that expression of Nodal ligands in the dorsal-vegetal region before the MBT allows the embryo to ensure the dorsal fate of the area by 1) establishing the presence of dorsal identity prior to expression of ventralizing factors at the MBT and 2) jump-starting a Nodal feed-forward regulatory loop that guarantees a high dorsal level of Nodal signaling by late blastula stages. In conjunction with the establishment of poised chromatin architecture at organizer genes by preMBT dorsal β-catenin activity, this mechanism could promote the establishment of dorsal mesoderm and the embryonic organizer. Therefore, in the context of *xnr5* and *xnr6* expression, preMBT transcription plays a critical role in patterning the embryo.

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